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REVIEW ARTICLE AGROBIOLOGICAL RECORDS
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USE OF MEDICINAL PLANTS AS ALTERNATIVE FOR THE CONTROL OF INTESTINAL PARASITOSIS: ASSESSMENT AND PERSPECTIVES

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ABSTRACT

Intestinal parasitosis is a significant health problem for animal husbandry, generating economic losses. In the treatment of helminths, synthetic anthelmintics have long been used. However, cases of parasitic resistance to these anthelmintics have been reported worldwide. Solutions have been proposed to solve this problem. Among them is the use of medicinal plants with anthelmintic properties. This work suggests synthesizing the work carried out on evaluating the anthelmintic properties of medicinal plants used to treat intestinal parasitosis of small ruminants. According to the results of the ethnobotanical surveys reported, several medicinal plants are used by the populations to treat intestinal parasitosis of small ruminants. Evaluations of the anthelmintic properties in vitro and or in vivo of some of them have confirmed their potential to be used as an alternative for controlling intestinal parasitosis. However, these results obtained depend on the organ of the species used, the type of extract, and the application dose. Tannins, flavonoids, and essential oils are the secondary metabolites responsible for the anthelmintic activity of these medicinal plants with anthelmintic potential. The efficacy of a plant extract (or powder) also depends on the parasite used in the tests. The results of previous studies confirm the use of medicinal plants in the fight against intestinal parasitosis.

***Keywords*:** Anthelmintic, Intestinal parasitosis, Medicinal plants, Resistance

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1. INTRODUCTION

Gastrointestinal parasitosis remains a real health problem for small ruminant livestock, especially because of the resistance developed by parasites against synthetic anthelmintics (Akoto et al. 2019; Nath et al. 2019; Rehman et al. 2021). Indeed, this state of affairs compromises the effectiveness of these drugs. Small ruminant breeding is an income-generating activity and therefore contributes to the national economy in developing countries like Benin. In pastures, small ruminants are more vulnerable to strongyles *Oesophagostoma venulosum*, *Chabertia ovina* (superfamily Strongyloidea), *Cooperia curticei*, *Nematoridus battus*, *Trichostrongylus axei*, *Trichostrongylus colubriformis* and *Haemonchus contortus* (superfamily Trichostrongyloidea). The species of the superfamily Trichostrongyloidea are distinguished by their absent or rudimentary oral capsule and a highly developed copulatory bursa and are the most pathogenic strongyles (Aguerre 2019). Infections by these gastrointestinal parasites, lead to weight loss of animals, poor wool quality, generating economic losses and can compromise food security (Alowanou et al. 2015). It is then important to use other solutions to control gastrointestinal parasites. Among the proposed populations is the use of medicinal plants with anthelmintic potential. The knowledge about use of medicinal plants is a tradition that is perpetuated from one generation to another. Ecological zone, level of education and ethnicity are factors related to the knowledge and use of medicinal plants by herders in Benin (Dassou et al. 2015). As a result of exchanges with the populations, medicinal plants have been listed and some

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have already been studied to evaluate their effectiveness on gastrointestinal parasites. However, the following question must be asked. What are or will be the challenges related to the use of medicinal plants in the treatment of gastrointestinal parasitosis? The research will have found its outcome when the breeder will have found relief or satisfaction through an effective treatment of gastrointestinal parasitosis of these animals at lower cost. It can be translated by the indication of effective plants and or the provision of improved traditional medicines accessible and at lower cost. It is with regard to all these observations, that the present study proposes to make the synthesis of the works in order to bring out the limits for a better use of the medicinal plants endowed with anthelmintic properties.

1.2. Parasite resistance to synthetic anthelmintics

Parasite resistance is defined as: the ability of a parasite population to resist concentrations of antiparasitic drugs that are usually lethal to individuals of that species. It is a mutational preadaptation whose determinism is genetic and inherited (Okombe and Pongombo 2013). Isotype 1 of the beta-tubulin gene SNPs is thought to be linked to helminth resistance to benzimidazole (Furtado et al. 2019). Factors favoring parasite resistance to anthelmintics are: frequency of treatments per time interval, adherence to dosage (under-dosing), exclusive use of a single anthelmintic or family of anthelmintics, grazing management (small ruminants). Other so called intrinsic factors are related to the life cycle of the parasites (short cycle), their prolificity (high) and pathogenicity (highly pathogenic) (Aguerre 2019). *Haemonchus contortus* parasite of small ruminants has a life cycle of 20 days and the female has the capacity to lay 5,000 to 10,000 eggs per day. It lodges in the abomasum and is hematophagous (Emery et al. 2016). These characteristics justify its resistance to synthetic anthelmintics. Resistance of other helminths to synthetic anthelmintics has also been reported. The Fig. 1 shows the three main lines of thought and alternative solutions in solving the difficulties related to the resistance of parasites to synthetic anthelmintics in the management of gastrointestinal parasitosis of small ruminants.

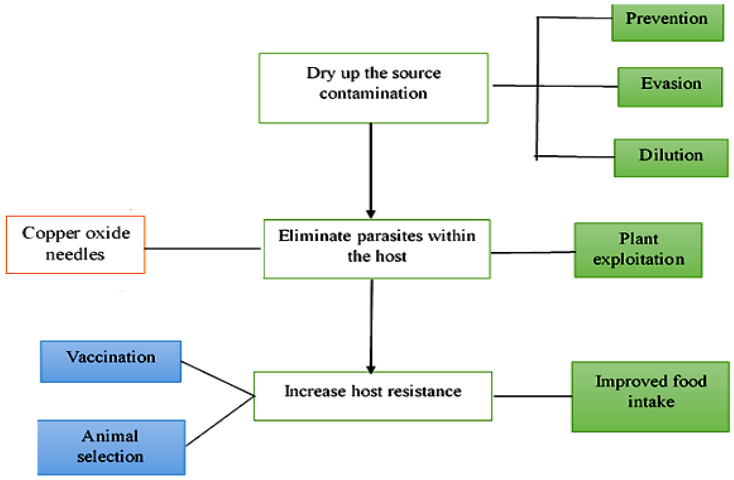

Fig. 1: Alternative solutions for the control of gastrointestinal parasitosis in small ruminants.

The interest of drying up the source of contamination is to block the biological cycle of the parasites in order to control the infestation of the pasture and reduce new infestations. It is done through prevention, i.e. healthy animals on clean pastures. In case of infections by gastrointestinal parasites, the treated animals will be transferred from contaminated pastures to clean pastures: this is the escape and finally the dilution. Another solution is to improve the host's resistance to parasites. This can be done by selecting NGIs resistant animals, by vaccination and or by improving the host's feed ration. The third alternative is to eliminate parasites within the host through the use of medicinal plants with anthelmintic properties (Kuiseu et al. 2021).

The use of medicinal plants with anthelmintic potentiality is the most promising alternative. The exploitation of medicinal plants will limit the presence of residues in the environment or in consumer products and would be more accessible to the population.

1.3. Medicinal plants with anthelmintic potential

Several medicinal plants are used by the populations in the treatment of gastrointestinal parasitosis of small ruminants (Table 1). These results are obtained on the basis of the endogenous knowledge of the populations. The knowledge and use of medicinal plants are a kind of tradition that is transmitted from one generation to another. Some of these plants with anthelmintic potential have been studied for their activities *in vitro* and / or *in vivo*. It is evaluated through tests on the different life stages (eggs, larvae and adult worms) of these parasites. *Haemonchus*

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contortus is the most studied nematode of small ruminants and is justified by its veterinary importance. It is one of the most pathogenic parasites infecting small ruminants. In Benin, its prevalence is 55.56%. (Attindehou et al. 2012). It is a hematophagous nematode that lodges in the abomasum of animals and 2000 females can puncture 30 ml of blood per day from a sheep (Coyne and Smith 1992). Also, cases of resistance of the *Haemonchus contortus* parasite to synthetic anthelmintics have been reported.

Table 1: List of some medicinal plants traditionally used in the treatment of gastrointestinal parasitosis and tested in small ruminants

Family	Species	Pararsite studied	In vitro	In vivo	Comments	References
Annonaceae	*Annona senegalensis*	*H. contortus*	-	+	The leaves of *A. senegalensis* appear to be more effective than other parts of the plant. The parasite spectrum sensitive to the different extracts of *A. senegalensis* is composed of strongyles (*H. contortus, Trychostrongylus sp* ...) and probably coccidia	Nguessan et al. (2017)
Anacardiaceae	*Spondias mombin*	*H. contortus*	+	+	Ethanolic and methanolic extracts of *S. mombin* leaves inhibited the migration of L3 larvae and the motility of adult *H. contortus* worms. However, the adult worms were significantly more sensitive after 6 hours of exposure to ethanolic extracts than methanolic extracts. *In vivo*, *S. mombin* leaf powder reduced the rate of egg excretion in parasitized animals.	Akouedegni et al. (2019)
Bignoniaceae	*Newbouldia laevis*	*T. colubriformis*	+	+	The tannins contained in the leaves of *N. laevi* are responsible for inhibiting the migration of L3 larvae. After digestion in the rumen the leaves of *N. laevis* would retain their anthelmintic activities	Olounladé et al. (2017); Sidi et al. (2017b)
Caesalpinioideae	*Cassia alata*	*H. contortus*	+	-	The aqueous and hydroacetonic extract of *C. alata* leaves have ovicidal and larvicidal activities. The leaves are rich in steroidal and triterpenic compounds, anthracenosides, saponosides, polyphenols (tannins)	Tianhoun et al. (2020)
	Cassia italica	*H. contortus*	+	-	The aqueous and ethanolic extracts of the leaves of *S. italitica* contain polyphenols (flavonoid tannins). Aqueous and ethanolic extract of *S. italica* exhibited evidence of *in vitro* anthelmintic activity on eggs; infective larvae L3 and against adults of *H. contortus*.	Yongwa et al. (2020)
Chenopodiaceae	*Chenopodium ambrosioides*	*H. contortus; O. colombianum*	+	-	The leaf powder of *C. ambrosioides* is rich in tannins, flavonoids, alkaloids, saponosides. aqueous extract of the leaves of *C. ambrosioides* has strongylicidal effects against *H. contortus* and *O. columbianum*.	Maiga et al. (2020)
Combretaceae	*Combretum glutinosum*	*H. contortus*	+	+	The leaves of *C. glutinosum* are rich in tannins, saponins flavonoids, alkaloids, glycosides, anthraquinones terpenes, reducing compounds. Acetonic and methanolic extracts of *C. glutinosum* leaves inhibited egg hatching, larval migration and adult worm motility.	Alowanou et al. (2019b)
Euphorbiaceae	*Bridelia ferruginea*	*H. contortus*	+	+	The leaves of *B. ferruginea* are rich in tannins, saponins, flavonoids, alkaloids, glycosides, anthraquinones, terpenes, reducing compounds. Acetonic and methanolic extracts of *B. ferruginea* leaves inhibited egg hatching, larval migration and adult worm motility. Acetonic extract and tannins from *B. ferruginea* leaves inhibited the emergence *of H. contortus* L3 larvae	Alowanou et al. (2019a); Tchetan et al. (2020)
Fabaceae	*Pterocarpus erinaceus*	*H. contortus*	+	-	The leaves of *P. erinaceus* are rich in anthracene derivatives, tannins, flavonoids, saponosides. Acetonic and methanolic extracts inhibited the migration of L3 larvae and the motility of adult *H. contortus* worms	Dedehou et al. (2014)

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Lythraceae	*Punica granatum*	*H. contortus*	+	-	The roots of *P. granatum* contains Alkaloid, Saponin, Tannins, Flavonoids, Glycosides and Phenol. Methanolic extract of P. granatum inhibited egg hatch and motility of adult *H. contortus* worms	Ahmed et al. (2020)
Mimosoideae	*Parkia biglobosa*	*H. contortus*	+	-	Fruit pods are rich in alkaloids and tannins. The acetone extract of *P. biglobossa* fruit pods was more reactive on the migration of L3 larvae than the methanolic extract	Dedehou et al. (2014)
	Albizia adianthifolia	*H. contortus*	+	-	The hydroalcoholic extract of *A. adianthifolia* showed larvicidal activity on L3 larvae of *Haemonchus contortus*	Mesmin et al. (2018)
Moraceae	*Ficus exasperata*	*H. contortus*	+	-	Aqueous extract of *F. exasperarata* inhibited adult worms motility	Houngnimassoun et al. (2017)
	Ficus lutea	*H. contortus*	+	-	The hydroalcoholic extract of *F. lutea* showed larvicidal activity on L3 larvae of *H. contortus*	Mesmin et al. (2018)
	Morus Mesozygia	*H. contortus*	+	-	The hydroalcoholic extract of *A. adianthifolia* showed ovicidal activity on the larval eggs of *H. contortus*	Mesmin et al. (2018)
Rubiaceae	*Mitragyna inermis*	*H. contortus ; T. colubriformis*	+	+	The leaves of *M. inermis* are rich in tannins, saponins, flavonoids, alkaloids, glycosides, anthraquinones, terpenes, reducing compounds. Aqueous extract of *M. inermis* leaves inhibited the motility of adult *T. colubriformis* worms. Acetonic and methanolic extracts of *M. inermis* leaves inhibited egg hatching, larval migration and adult worm motility. Acetonic extract and tannins from the leaves of *M. inermis* inhibited the emergence of L3 larvae of *H. contortus*	Alowanou et al. (2019a, b); Tchetan et al. (2020); Toklo et al. (2021)
Rutaceae	*Zanthoxylum zanthoxyloïdes*	*H. contortus; T. Colubriformis*	+	+	The tannins contained in the leaves of *Z. zanthoxyloïdes* are responsible for inhibiting the migration of L3 larvae. After digestion in the rumen the leaves of *N. laevis* would retain their anthelmintic activities	Olounladé et al. (2017); Azando et al. (2017 Sidi et al. (2017b)
Combretaceae	*Terminalia Catappa*	*H. contortus*	+	-	Dichloromethane extract of *T. catappa* leaves inhibited the hatching of *H. contortus* eggs more than methanolic extract	Olukotun et al. (2018)
Asteraceae	*Artemisia herba-alba*	*H. contortus*	+	-	The flowers and aerial part of *A. herba-alba* contains alkaloid, saponin, tannins, flavonoids, glycosides and phenol. The methanolic extract of *A. herba-alba* inhibited egg hatch and motility of adult *H. contortus* worms	Ahmed et al. (2020)
Zingiberaceae	*Curcuma longa*	*H. contortus*	+	-	The hydroalcoholic extract of *C. longa* reduced the rate of egg excretion from infected animals	Nath et al. (2019)
Celastraceae	*Maytenus senegalensis*	*H. contortus*	+	-	Tannins are responsible for the anthelmintic activities of the aqueous extract of *M. senegalensis*	Zangueu et al. (2018)
Lamiaceae	*Thymus vulgaris L.*	*H. contortus*	+	+	Thymol, the main compound in the essential oil of *Thymus vulgaris* L, is believed to be responsible for its anthelmintic activity.	Ferreira et al. (2016)

Egg hatch tests, inhibition of larval migration, and adult worm motility are all techniques used to evaluate the efficacy of extracts on gastrointestinal nematodes. The egg hatch test is a test that evaluates the ability of an extract to act on egg hatching. The inhibition of the survival of adult worms induced by an extract is an important factor in the control of these parasites. Indeed, the disruption of their motility would result in difficulties in feeding (thus less blood depletion for hematophagous parasites) and mating (thus less egg excretion) (Zinsou 2015; Sobhy et al. 2021). The decrease in larval migration, could disrupt their settlement in the mucosal wall of the digestive tract (Dedehou et al. 2014; Tchetan et al. 2020). The tests performed *in vitro* allow a rapid evaluation of the anthelmintic properties of the different plants tested and the analysis of the mechanisms of action of these plants on the parasites (Akouedegni et al. 2019). *Z. zanthoxyloides, N. laevis, S. monbin, C. italitica, B. ferruginea, A. herba-alba, C. longa* are some of the species that demonstrated their anthelmintic activities *in vitro* through egg hatching, larval migration inhibition and adult worm motility tests. Within the organism, several other components such as the

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metabolism of the medicinal plant (or its extract), the availability and concentration of the active principle(s) can influence the activity of the medicinal plant (or its extract), hence the importance of *in vivo* tests. Also, the doses and frequencies of ingestion of anthelmintic plants are important factors to control for good efficacy, as the concentrations in the digestate can vary from one portion of the digestive tract to another (Azando et al. 2011). Thus, results obtained during *in vitro* testing may not match the *in vivo* test (Akouedegni et al. 2019). *In vivo* tests performed on artificially infested sheep have demonstrated the therapeutic effect of extracts of *Z. zanthoxyloides, N. laevis, S. monbin, M. inermis A. senegalensis* (Azando et al. 2011; Azando et al. 2017; Akouedegni et al. 2019). *S. monbin* leaf powder and *Annona senegalensis* leaf extract administered to naturally infested sheep have been shown to be effective in reducing egg shedding with selectivity (Ademola et al. 2005; Nguessan et al. 2017). That is, the extracts are more active on some parasites than others. The aqueous extract of *Annona senegalensis* would not be effective on Taenia spp. (Nguessan et al. 2017). *Z. zanthoxyloides* and *N. laevis* powders would be more effective on *H. contortus* eggs than on *T. colubriformis* and *O. columbianum* eggs (Azando et al. 2011).

Table 2: List of some medicinal plants traditionally used in the treatment of gastrointestinal parasitosis and not yet tested in small ruminants

Family	Species	References
Acanthaceae	*Nelsonia canescens (Lam.)*	Dassou et al. (2015); Ogni et al. (2014)
Alliaceae	*Allium sativum*	Dassou et al. (2012); Ogni et al. (2014)
Amaranthaceae	*Amaranthus spinosus*	Ogni et al. (2014)
Anacardiaceae	*Anacardium occidentale*	Ogni et al. (2014)
	Lannea acida	Garba et al. (2019)
	Mangifera indica	Dassou et al. (2012); Ogni et al. (2014)
	Sclerocarya bierrea	Garba et al. (2019)
Apocynaceae	*Pleiocarpa pycnantha*	Dassou et al. (2012); Ogni et al. (2014)
Asclepiadaceae	*Leptadania hastata*	Garba et al. (2019)
Bignoniaceae	*Kigelia Africana*	Garba et al. (2019)
Bombacaceae	*Adansonia digitata*	Dassou et al. (2012); Ogni et al. (2014); Garba et al. (2019)
	Ceiba pentandra	Kabore et al. (2007)
Cachlospermaceae	*Cochlospermum planchonii*	Dassou et al. (2012); Ogni et al. (2014); Garba et al. (2019)
Caesalpinioideae	*Cassia sieberiana*	Dassou et al. (2012); Garba et al. (2019)
	Tamarindus indica	Ogni et al. (2014)
Capparaceae	*Boscia salicifolia*	Garba et al. (2019)
	Boscia senegalensis	Garba et al. (2019)
	Cadaba farinosa	Garba et al. (2019)
	Crateva adansonii	Dassou et al. (2012); Ogni et al. (2014)
	Maerua crassifolia	Garba et al. (2019)
Caricaceae	*Carica papaya*	Dassou et al. (2012); Attindehou et al. (2012); Sidi et al. (2017a); Islam et al. (2019)
Celtidaceae	*Celtis integrifolia*	Garba et al. (2019)
Cochlospermaceae	*Crotalaria rotusa*	Garba et al. (2019)
	Pilostigma reticulatum	Garba et al. (2019)
Combretaceae	*Anogeissus leiocarpa*	Dassou et al. (2012); Ogni et al. (2014); Garba et al. (2019);
	Combretum micranthum	Garba et al. (2019)
	Guiera senegalensis	Garba et al. (2019)
	Pteleopsis suberosa	Dassou et al. (2012); Ogni et al. (2014)
	Terminalia avicennioides	Dassou et al. (2012);
	Terminalia macroptera	Dassou et al. (2012); Ogni et al. (2014)
Convolvulacea	*Ipomoea asarifolia*	Dassou et al. (2012); Ogni et al. (2014)
	Ipomoea batatas	Dassou et al. (2012)
	Ipomoea eriocarpa	Dassou et al. (2012)
Crassulaceae	*Kalanchoe crenata*	Dassou et al. (2012)
Cucurbitaceae	*Adenopus breviflorus*	Dassou et al. (2012)
	Cucumis melo	Attindehou et al. (2012)
	Momordica charantia	Dassou et al. (2012)
Euphorbiaceae	*Croton zambesicus*	Garba et al. (2019)
	Euphorbia kamerunica	Dassou et al. (2012); Ogni et al. (2014)
	Jatropha curcas	Attindehou et al. (2012)
	Phyllanthus amarus	Attindehou et al. (2012)
	Acacia albida	Garba et al. (2019)
	Acacia nilotica	Garba et al. (2019)

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Fabaceae	*Cajanus cajan*	Attindehou et al. (2012)
	Gliricidia sepium	Attindehou et al. (2012)
	Tephrosia purpurea	Garba et al. (2019)
Lamiaceae	*Ocimum canum*	Attindehou et al. (2012)
	Ocimum gratissimum	Attindehou et al. (2012)
Leguminosae	*Caesalpinia bonduc*	Dassou et al. (2012)
	Caesalpinia pulcherrima	Dassou et al. (2012)
	Detarium microcarpum	Dassou et al. (2012)
	Isoberlinia doka	Dassou et al. (2012); Ogni et al. (2014)
	Prosopis africana	Dassou et al. 2012; (Garba et al. 2019)
Lythraceae	*Lawsonia inermis*	Garba et al. (2019);
Meliaceae	*Khaya senegalensis*	Dassou et al. (2012); Garba et al. (2019)
	Trichilia prieuriana	Attindehou et al. (2012)
	Azadirachta indica	Dassou et al. (2012); Attindehou et al. (2012); Ogni et al. (2014);
Menispermaceae	*Rhigiocarya racemifera*	Dassou et al. (2012); Ogni et al. (2014)
Moraceae	*Ficus unibellata*	Attindehou et al. (2012)
Moringaceae	*Moringa oleifera*	Dassou et al. (2012); Attindehou et al. (2012); Sidi et al. (2017a); Ogni et al. (2014)
Myrtaceae	*Psidium guajava*	Dassou et al. (2012); Attindehou et al. (2012); Ogni et al. (2014); Silva et al. (2020)
Olacaceae	*Ximenia americana*	Dassou et al. (2012)
Phytolaccaceae	*Petiveria alliacea*	Attindehou et al. (2012)
Poaceae	*Eragrostis tremula*	Garba et al. (2019)
	Zea mays	Kpabi et al. (2020)
Polygalaceae	*Securidaca longepedunculata*	Garba et al. (2019)
Rhamnaceae	*Ziziphus mauritiana*	Garba et al. (2019)
Rubiaceae	*Gardenia erubescens*	Kabore et al. (2007)
	Macrosphyra longistyla	Dassou et al. (2012)
	Sarcocephalus latifolius	Dassou et al. (2012); Ogni et al. (2014)
	Spermacoce stachydea	Garba et al. (2019)
Rutaceae	*Citrus limon*	Dassou et al. (2012)
	Citrus sinensis	Ogni et al. (2014)
	Harrisonia abyssinica	Attindehou et al. (2012)
Salvadoraceae	*Salvadora persica*	Garba et al. (2019)
Scrophulariaceae	*Striga hermonthica*	Garba et al. (2019)
Zygophyllaceae	*Balanites aegyptiaca*	Garba et al. (2019)

1.4. Secondary metabolites responsible for the anthelmintic activity of medicinal plants

The presence of bioactive substances such as flavonoids, alkaloids, tannins and essential oils confer anthelmintic properties to medicinal plants (Williams et al. 2014; Azando et al. 2017; Olounladé et al. 2017; Saha and Lachance 2020; Castro et al. 2021), Tannins reportedly inhibit larval migration and egg hatching (Olounladé et al. 2017; Tianhoun et al. 2020; Tchetan et al. 2020). Tannins will bind to free proteins in the cuticle which will then result in physiological alteration of motility, nutrition, absorption and reproduction (Githiori et al. 2006). Fig. 2 describes the mechanisms and sites of action of secondary metabolites of medicinal plants with anthelmintic properties.

1.5. Factors that may influence the anthelmintic activity of medicinal plants

The results obtained on the effectiveness of plant extracts on gastrointestinal parasites, depend on several parameters such as: the species part used; the type of extract; the dose of extract applied, and the parasite used (life stage). According to the work of Akouedegni et al. (2019), the ethanolic extract of *S. monbin* leaves is more effective on larval motility than the methanolic extract. Similar observations are reported by Ademola et al. (2005), according to which the aqueous extract of the same *S. monbin* plant is more effective on larvae than the ethanolic extract. The acetone extract of *P. biglobossa* is more effective on larval migration than methanolic extract (Dedehou et al. 2014); the acetonic extract of *Cassia alata* is more effective than the aqueous extract on egg hatching (Tianhoun et al. 2020). This difference in the activity of one extract of the same plant compared to another would be related to the presence or absence of certain secondary metabolites in one extract compared to the other (Tianhoun et al. 2020). Indeed, depending on affinities, a solvent has the potential to extract in majority certain secondary metabolites. However, the production of secondary metabolites in plant species is correlated to geographical, climatic and genetic factors of the species (Maiga et al. 2020). Essential oils extracted from medicinal plants are also subject to much variation in their chemical compositions. They can vary depending on the geographical site,

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time of day, season and genetic factors specific to the species itself. For a better use of anthelmintic medicinal plants, it is therefore necessary to master the factors of variation of secondary metabolites responsible for anthelmintic activities. It is also important to focus on the bioavailability of the species for a better exploitation of these medicinal plants.

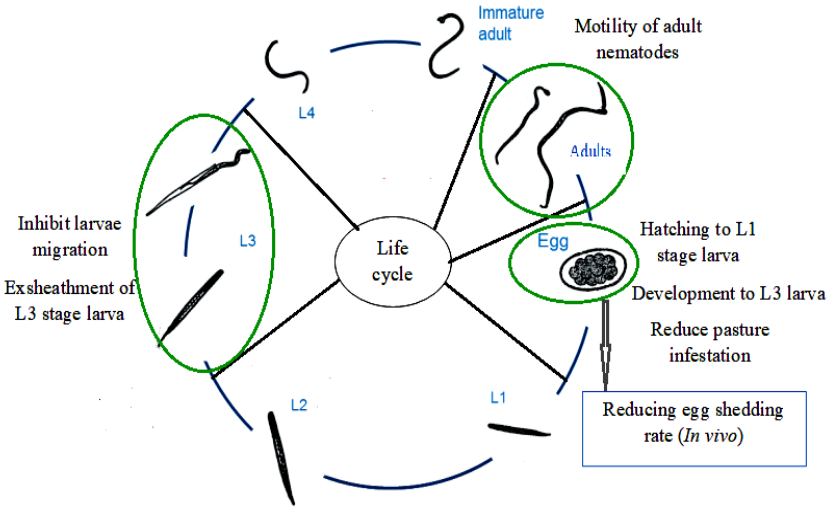

Fig. 2: Potential mechanisms of action of secondary metabolites (essential oil; tannins flavonoids, alkaloids, phenol) from medicinal plants on gastrointestinal parasites of small ruminants.

Conclusion: Through previous studies, medicinal plants have demonstrated their ability to be used in the control of gastrointestinal parasitosis in small ruminants. However, the anthelmintic activities of these plants depend on several factors that must be controlled. The secondary metabolites: flavonoids, alkaloids, tannins and essential oils responsible for the anthelmintic activity of medicinal plants are subject to variations according to geographical and intrinsic factors of the plants. Also, the secondary metabolites present in a given medicinal plant extract depend on the solvent used for extraction. Anthelmintic medicinal plants (or their extracts) do not have the same efficacy on parasitic nematodes, and small ruminants and even humans are often confronted with polyparasitism. In short, anthelmintic medicinal plants are good candidates in the fight against intestinal parasitosis.

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EPIDEMIOLOGICAL SURVEY OF TRYPANOSOMIASIS IN THE DROMEDARY CAMELS RAISED IN DERA ISMAIL KHAN, KHYBER PAKHTUNKHWA, PAKISTAN

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ABSTRACT

A one-year epidemiological survey was conducted to determine the age, sex, and season-wise prevalence of Trypanosomiasis in dromedary camels (Camelus dromedarius) at Dera Ismail Khan region of Khyber Pakhtunkhwa, province of Pakistan. A total of 600 blood samples were parasitological examined, of which 300 were male and 300 were females. Camels have grouped age-wise as Adults (<2 years age) and calves (>2 years age), sex-wise as; male and female, while kept the ratio of animals in both age and sex group, were kept nearly 1:1. Season-wise prevalence was recorded by splitting the data into four seasons: pre-monsoon, Monsoon, Post-Monsoon, and winter. The samples were examined microscopically using thin, thick, and wet blood film to identify Trypanosoma evansi based on morphological characteristics. An overall prevalence was 6.67%. Age-wise prevalence was high at 9.06% in adults and low at 4.30% in calves ($P<0.05$). Sex-wise, the infestation was observed as high 7.66% in females and low 5.66% in male camels ($P>0.05$). Among four seasons of the year, the highest prevalence, 11.18%, was observed in the rainy season of monsoon, followed by 8.45, 7.85, and 0% in post-monsoon, pre-monsoon, and winter, respectively ($P<0.05$). All the results were statistically analyzed on SPSS using the Pearson Chi-Square test. This survey showed that age and season have a significant effect while sex has a non-significant effect on the prevalence of Trypanosomiasis in dromedary camels.

***Keywords*:** Trypanosomiasis, age, sex, season, dromedary camels.

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1. INTRODUCTION

Livestock industry has a big share in the economy of Pakistan, contributing about 11.5% to the total Gross Domestic Products (GDP). About 8 million families are earning 40% of their livelihood from livestock sector (Anonymous 2020-2021). In Pakistan, small and large ruminants including camels are mainly reared for meat and milk production (Ali et al. 2016; Ali et al. 2017). Camel (Camelus dromedarius) is a single humped animal an important multipurpose animal which is raised in arid and semi-arid areas of world. The best source of livelihood of people of desert areas is the camel in Asia, Middle East and Africa (Hussain et al. 2018; AL-Samawy et al. 2019; Gherissi et al. 2020; Hussain et al. 2021). Total camel population of word is about 25.89 million, having 89% single humped animals whereas 11% are double humped (*Camelus bacterianus*) (FAO 2013). Camels in the agriculture-based country like Pakistan are contributing 1.81% towards total milk production by supplying 932000 tons of milk per annum (Economic Survey of Pakistan 2020). Diseases like bacterial, viral and parasitic/protozoal infections have always remained a constant threat to the productivity of animals (Batool et al. 2019; Chemweno et al. 2019).

Trypanosoma evansi (*T. evansi*) is hemoflagellate parasite of order Kinetoplastida and family Trypanosomatidae which replicates in blood and other body fluids (Alsaad 2021). It causes a widely spread endemic disease called "Surra" in camels, various domestic as well as wild animals throughout the world (Elhaig et al. 2013; Rodríguez et al. 2012; Shahid et al. 2013; Chau et al. 2016). Trypanosomiasis was discovered in India more than a hundred years ago by Evans (1880), who detected it in horses, mules and camels causing a disease which was named as "Surra". Including different mammals, humans are also affected (Coura and Borges-Pereira 2010; Dyary et al. 2014). The case of human trypanosomiasis has been reported from Southeast Asia (Chau et al. 2016). It is mechanically transmitted by biting flies (*Tabanus, Stomoxys* and *Liperosia*) in which the protozoans undergo a biological cycle (Yadav et al. 2011). Animals suffering from these protozoan parasites usually suffer from induce fever, dullness, weakness, depression, and anemia. Also, these parasites are responsible for economic losses in terms of poor production (low

Khan SZ, Umm-e-Aimen, Rizwan M, Ali A, Khan I, Safiullah, Abidullah, Imdad S, Waseemullah and Khan A, 2022. Epidemiological survey of trypanosomiasis in the dromedary camels raised in Dera Ismail Khan, Khyber Pakhtunkhwa, Pakistan. Agrobiological Records 7: 10-17. https://doi.org/10.47278/journal.abr/2021.010

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meat and milk production, low fertility, and less draught power available) and sometimes abortion or death in case of no treatment (Enwezor and Sackey 2005). Trypanosomiasis in camels is characterized by reduced appetite, low water intake, disappearance of hump with disease progress, dull and rough hair coat with loss of hairs at tail. Odema of under belly is visible especially in the morning, pregnant females may abort, and newborn calves of infected calves usually die. There is pallor of mucous membranes of the eyes, a fluctuating temperature with initial peaks of up to 41°C, and urine usually has characteristic smell (Köhler-Rollefson et al. 2001). Animals become emaciated and die within 2-4 months due to anemia, circulatory disturbances and myocardial damages leading to congestive heart failure. The camel may live up to four years with sub-clinical infestation and some may eventually recover and eliminate the parasite. Direct losses due to trypanosomiasis are estimated at US$ 1-1.2 billion each year. The total losses for the total tsetse fly infested land in terms of Agricultural Gross domestic product (GDP) are US$ 4.75 billion per year (FAO 2000).

Trypanosomiasis has a wide range of distribution throughout tropical and sub-tropical regions of the world. The disease is most important single cause of economic losses in camel rearing areas (Angara et al. 2012), causing morbidity of up to 30% and mortality of around 3% (Luckins 1988). Surveys in various tropical areas have shown a definite correlation between seasonal outbreaks of *T. evansi* infections and the increase in number of Tabanus during rainy season (Mahmoud and Gray 1980). Camel Trypanosomiasis is present in India, Myanmar, Bhutan, Mongolia, Malaysia, Cambodia, China, Thailand, Russia, Nepal, Vietnam, Philippines, Indonesia, South and Latin America and Europe with worldwide distribution (Reid 2002; Mihret and Mano 2007; Konnai et al. 2009; Sood et al. 2011; Salim et al. 2011; Ismael et al. 2014; Kumar et al. 2017; Boushaki et al. 2019). Recently the disease has also been reported in the Canary Island, Spain as well as France (Gutierrez et al. 2000). This disease is a serious concern as it is difficult to treat and non-availability of vaccine (Metwally et al. 2021). The disease replication is supported with environmental and climatic conditions of the regions and also presence of the vectors in the area play an important role in the spread of the disease. Environmental high temperature and relative humidity favors the division of its vectors and thus increases the chances of the trypanosomiasis (Desquesnes et al. 2013).

According to Livestock census conducted in 2006, there are 12930 camels in district Dera Ismail Khan, those are mostly used to pull carts or plough fields. Present study was conducted to determine the age, sex and season wise prevalence of Trypanosomiasis in the dromedary camel population reared in rural areas of District Dera Ismail Khan.

2. MATERIALS AND METHODS

2.1. Sample area

Samples were collected from 20 different villages of D.I. Khan (Fig. 1) including Garrah Rehman, Kulachi, Hathala, Draban Kalan, Chudhwan, Musazzai, Hassani, GarrahJat, Garrah Ali, Garah Meharban, Lar, Kala Ghore, Kot jaiee, Abdul Khel, Shah Hassan Khel, Panyalla, Wanda Madad, Wanda Kali, Yarak and Rori Khel, where dromedary camels are raised. The area covers pane land and somewhere sandy area.

Fig. 1: Map of Pakistan (right side) showing all provinces and on the left side is Khyber Pakhtunkhwa Province and Dera Ismail Khan (study area in red color).

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2.2. Sample Collection

During field survey, samples were collected randomly from the animals of respective area including both the healthy as well as morbid animals, if any. For sample collection, regular field visits to the respective villages were conducted on monthly basis. A total of 600 blood samples were collected form Jugular vein/ear vein, after disinfecting the site with 70% alcohol. Punctured into 5mL EDTA coated vacutainer tubes, properly labeled and kept in ice box. Both the thick and thin blood smears were made at the spot of collection. Thin smears were fixed with absolute methanol for 5min. Smeared slides were also labeled and placed in ice box. Ice box containing the seared slides and blood samples were transported to Parasitology laboratory at Veterinary Research and Disease Investigation Center, D.I. Khan for further processing and examination.

2.3. Sample processing

Wet film technique was used to observed Trypanosoma by their waving flagellum movement (Murry et al. 1979). From each camel thin smears of fresh blood were prepared for the microscopic study and identification of flagella of trypanosomes (Hussain et al. 2018). Blood smears prepared were dried, fixed in ethanol (100%) and stained with Giemsa stain. For different morphological changes in erythrocytes and the presence of *Trypanosoma*, all the blood smears were carefully studied under light microscope (Hussain et al. 2018).

Stained Blood Smear (Both thick and thin) were used for detection and identification of Trypanosoma species based on their morphological characters Samples were parasitological examined under Oil emersion lens (100X). Morphological characteristics of *T. evansi* recognized from blood samples of infected camels following the methods described by Chandler and Read (1961), while the *Trypanosoma evansi* measurements were carried out following the method of Luckins (1992) and Smyth (1996).

2.4. Seasons Distribution

This study was carried out throughout the year; thus, a year was divided into four seasons, i.e., pre-monsoon (March to May), monsoon (June-September), post-monsoon (October and November) and winter (December-February).

2.5. Statistical Analysis

Data thus collected were subjected to statistical analysis. Camels were categorized into age-wise (>2 years: calves; <2 years: adult camels), sex-wise (male and female) and season-wise (four seasons). Data were reviewed by descriptive statistics using SPSS V21.0, (IBM SPSS Statistics 21, USA). Comparison between groups was evaluated using Pearson Chi-Square test (Petrie and Watson 1999). Any result variable was considered as significant at $P<0.05$.

3. RESULTS

Out of total camels (n=600) under study, 40 were positive with *T. evansi* showing overall prevalence of 6.67%. During the investigations, *T. evansi* was only specie recognized from the samples of infected animals. Age, sex and season-wise results of the study are presented in Tables 1-3, respectively. Despite its low sensitivity, as a routine diagnostic practice, parasitological examination of the samples was conducted due to non-availability of molecular testing facilities at the center. Morphological characteristics of *T. evansi* recognized from blood samples of infected camels. *Trypanosoma evansi* have slender body with centrally located nucleus, and subterminal kinetoplast with the length. *T. evansi* were measured which ranged from 15.30µm in length and 1.5 to 3µm in width.

Table 1: Age-wise prevalence of Trypanosomiasis in dromedary camels

Group	Age	Total camels	Infected Camels		Non-infected camels	
			No	%	No	%
Adult	<2 years	298	27	9.06	271	90.93
Calves	>2 years	302	13	4.30	289	95.70
Total		600	40	6.67	560	93.33

Data analysis by Chi-square test. χ^2 Value=4.772; df=1; P value=0.029.

Table 2: Sex-wise prevalence of Trypanosomiasis in dromedary camels

Sex	Non-infected camels	Infected camels		χ2 Value	P Value
		No.	%		
Male	283	17	5.66	0.964	0.326
Female	277	23	7.66		
Total	560	40	6.67		

Data analysis by Chi-square test. χ^2 Value=0.964; df=1; P value=0.326.

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Table 3: Season-wise prevalence of Trypanosomiasis in dromedary camels

Season	Non-infected Camels	Infected Camels	
		No.	%
Pre-Monsoon	191	15	7.85
Monsoon	170	19	11.18
Post-Monsoon	71	06	8.45
Winter	128	0	0
Total	560	40	6.67

Data analysis by Chi-square test. χ^2 Value=12.908; df=3; P value=0.005.

Prevalence of *T. evansi* infestation in adult camels (<2 years age) was 9.06% while in calves (>2 years age) was 4.30%. The results showed that adult age group was more affected than the calves. The difference in prevalence between age groups was found statistically significant (P<0.029) as shown in Table 1.

Prevalence of *T. evansi* infestation in more in male camels (5.66%) as compared to female camels (7.66%). The difference in prevalence of *T. evansi* between male and female dromedary camels was found non-significantly different (Table 2).

Camels (n=600) from those blood samples collected have distribution the highest in the pre-monsoon (34%) followed by the monsoon (32%), winter (21%) and least (13%) in post-monsoon (Fig. 2). Seasonal prevalence of *T. evansi* was found high 11.18% in monsoon, moderate 8.45% in post monsoon, low 7.85% in pre-monsoon while no case in winter season. The difference in prevalence among four seasons of the year was found statistically significant (P<0.05) as shown in Table 3.

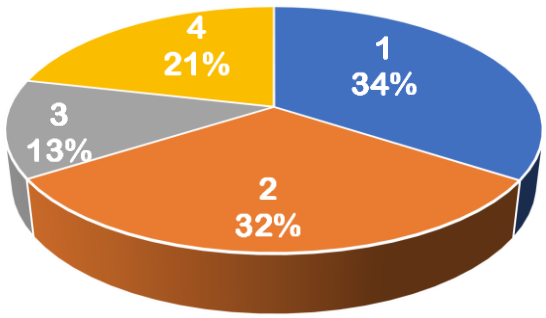

Fig. 2: Camels (n=600) distribution (%) among various seasons, i.e., 1) pre-monsoon, 2) monsoon, 3) post-monsoon and 4) winter from those samples were collected.

4. DISCUSSION

This is the first study on *Trypanosoma evansi* infection in Camelidae using parasitological examination in D.I. Khan, KP Province, Pakistan. Morphologically, *T. evansi* is an elongated blood parasite and measured 14-29µm in length and 1.5 to 3.45µm in width, with a nucleus containing a large central nucleolus (Dávila et al. 1998). According to Khalafalla and Al Mawly (2020), *Trypanosoma evansi* were characterized by slender body, centrally located nucleus, and subterminal kinetoplast with total length including free flagellum of 24.9µm (16.9–31.3µm), the body length of 16.4µm (7.9–25.1µm) and width 2.8µm (0.1–5.7µm). The free flagellum is 28.6µm long (1.1–14.1µm).

The prevalence of "Surra" in Dera Ismail Khan Region as 6.67% can be considered as low compared with other epidemic regions like Somalia (Baumann and Zessin 1992), Kenya (Njiru et al. 2001) and Egypt (Sobhy et al. 2017). Metwally et al. (2021) using molecular method (PCR), reported prevalence of Trypanosomiasis as 42.5% in Kingdom of Saudi Arabia, which is higher in comparison to the result of the present study. Mossaad et al. (2017) and Gerem et al. (2020) in their study reported that the prevalence of Trypanosomiasis is found lower on parasitological examination method as compared to the molecular techniques (PCR). Similar results were reported by Birhanu et al. (2015) and Sadek et al. (2021). Similarly, the prevalence was found 25% in northern part of Kingdom of Saudi Arabia (Al-Joaf Province) (Elwathig et al. 2016). Kasim (1984) and Hussein et al. (1991) reported the prevalence of Trypanosomiasis on microscopic study as 2 to 13.2% in the Saudi Arabia which lies in relation with the findings of this study. Another study conducted by Omer et al. (1998) revealed the prevalence as 5.5% in camel population of Saudi Arabia. Hussain et al. (2021) reported the prevalence as10.58% from Cholistan region of Province of Punjab, Pakistan which shows more prevalent nature of the disease in Cholistan desert as compared to the present study. This increase can be attributed to the harsh environmental conditions and higher number of vector population in the area as compared to

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the present study area. Ahmadi-Hamedani et al. (2014), also concluded that trypanosomiasis was present in 4.76% camels of Semnan, Iran. Khosravi et al. (2011) indicated that *T. evansi* was present in 2.1% camels of Rafsanjani, Kerman province, Iran. The results of this study are closer to the study of Hassan et al. (2006) in which 3.3 to 4% prevalence level was observed in Punjab region of Pakistan and also with Ahmadi-Hamedani et al. (2014) who noted 4.67% in Iran. However, the study revealed lower level of prevalence than earlier studies such as 13.2% by Hussein et al. (1991), 13.72% by Shah et al. (2004) and 11.5% by Bhutto et al. (2010). Factors like ecological variations and vector abundance may lie behind this difference.

This study showed higher rate of infection in adults than calves and the similar difference of 0% prevalence in calf (≤2 years) and 7.7% prevalence in young group (>2 years) was observed in Ethiopia (Kassa et al. 2011). It was also reported from Egypt that prevalence level of *T. evansi* in calves (≤4 years) was 14% and in adults (4< years) was 24-25% (Sobhy et al. 2017) showing higher infection level in adults and lower in calves. Hussain et al. (2016) reported that the difference between the adults and younger camels, but non-significant but older camels were infected more unlike the young camels. They attributed this difference towards the poor management, improper diet and transportation of heavy draught duty. Immunology could also be a factor for protecting the camel from *T. evansi* infestation (Hussen and Schuberth 2021). Similar results were mentioned by other researchers (Shah et al. 2004). Contrary to the results of the present study, relatively higher prevalence of trypanosomiasis has been reported in young camels as compared to adult ones (Kassa et al. 2011). The calves may be less infected due to their confinement at the stall where the vector population could be less as compared to meadow lands where adults camels are grazed by pastoralists.

The higher infection rate in females could be attributed to stress of lactation and successive pregnancies (Bhutto et al. 2010). A higher infection rate (15.68%) in females were relatively lower in males (11.76%) was also found (Shah et al. 2004). Iranian study reflects that in female the loss increases as a result of abortions. The rate of abortion in pregnant females was 16.4% in South Khurasan and 26.3% in Sistan and Baluchistan provinces of Iran (Nazem et al. 2020). Higher infection of *T. evansi* in females (22.9%) and lower in males (14.1%) has also been reported from Egypt (Sobhy et al. 2017). A previous report from Ethiopia (Kassa et al. 2011) showed higher prevalence of *T. evansi* (6.8%) in males than females (4%) which is unlike to the present study. This variation could be due to stress of loading and transportation fatigue acquiring probability of higher infection in male camels. Sobhay et al. (2017) reported higher prevalence in male camels (22.9%) as compared to females (14.1%) on microscopic parasitological examination. Similar data were recorded by Barghash et al. (2014), El-Naga and Barghash (2016) and Sobhy et al. (2017).

It has been unearthing by that the highest prevalence was found in the spring season (26.77%), followed by the summer (23.47%), winter (16%) and autumn (12.6%) using microscopic parasitological techniques. Similar results have also been reported by Sobhy et al. (2017). It was interesting to note that season-wise prevalence of *T. evansi* was related to the biting horseflies (Tabanus species) in the area. These flies are found in abundance during rainy seasons and lower in winter. Dera Ismail Khan District, Khyber Pakhtunkhwa province consists mostly of floodplain land situated at the western bank of the river Indus. The area is with extreme weather conditions during summer and winter. However, rainy season (monsoon) reduces the effect of scorching heat in summer. The high prevalence of Trypanosomiasis in rainy season could be linked with the higher vector population of Tabanus species (Kassa et al. 2011). Luckins (1988) was of the view that season has a direct effect on the distribution of biting flies, which are responsible for the mechanical transmission of *T. evansi*. The prevalence of these flies is higher in spring and summer season; hence the incidence rate is also found higher in these seasons. It was concluded that the best season for Trypanosomiasis is the Spring followed by the Summer that might be related to a higher risk of camels exposure to trypanosome infection due to the increased density of vector populations at this time of the year as reported before and coincides with Barghash et al. (2014). Apart from rainy season, water withholding capacity of soil and surface water pool where Acacia Senegal shrub grows in abundance are considered suitable factors for survival and propagation of vector (Kassa et al. 2011).

Conclusion: The overall prevalence of Trypanosomiasis in dromedary camels was determined to be 6.67% in D.I. Khan, KP Province, Pakistan. It was concluded that the age and seasons of the year significantly ($P<0.05$) affect the prevalence of Trypanosomiasis in dromedary camels, while the sex has no effect. However, the results cannot be same for all camel producing areas of the country as the ecological and geological factors attributed to the spread of disease vary from area to area within the same country.

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Authors Contribution: SZK, UEA, and MR planned the study. AA, IK, Safiullah, Abidullah, SI, Waseemullah and AK collected the samples and analyzed in the Laboratory. SZK intrepreted the data and all authors were invloved in the revision and approval of final version of the manuscript.

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HEMATOLOGICAL, SERUM BIOCHEMISTRY, HISTOPATHOLOGICAL AND MUTAGENIC IMPACTS OF TRICLOSAN ON FISH (BIGHEAD CARP)

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ABSTRACT

Agro-aquatic ecosystems are mainly and persistently exposed to various unwanted chemicals and pollutants due to indiscriminate use of agrochemicals such as pesticides, insecticides, heavy metals, germicides, drug residues, industrial wastes, and feed additives. Randomly kept 16 active fish (bighead carp) in four unlike groups (A to D). Blood and serum were collected on days 5, 10, and 15 of the experiment. Five fish from each group were slaughtered on day 15 of the trial to study histopathological alterations. Mild to moderate different physical ailments like jerking movement, erratic swimming, and mucous secretion from the mouth of fish kept in group D were observed after day 10 of post-exposure. Significantly, lower erythrocyte count, Hb, and hematocrit values while increased values of total white blood cells and neutrophil counts were recorded in fish of groups (C-D). Results on serum chemistry showed a significantly increased quantity of liver function tests (ALT and AST), renal functional tests (urea and creatinine), and cardiac biomarkers. Results on micronuclei and comet assay indicated an increased frequency of DNA damage. The frequency of nuclear and morphological variations in RBCs of fish of group (D) significantly increased compared to the control group. Results on microscopic levels exhibited different histopathological alterations in gills like twisting of secondary lamellae, uplifting of lamellae, lamellar disorganization, and necrosis of lamellar epithelial cells. In the liver, congestion, necrosis of hepatocytes, fatty infiltration, and brain necrosis and atrophy of neurons. Kidneys showed necrosis of tubules, increased urinary spaces, tubular necrosis in treated fish in groups (C-D) after day 10 of post-exposure. From the findings of our experimental research, we can suggest that triclosan causes toxic effects in bighead carp.

***Keywords*:** Bighead Carp, Triclosan, Blood, Serum, DNA damage, Histopathology

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1. INTRODUCTION

The existence and destiny of pharmaceutically active compounds in the marine ecosystem seems to be one of the emerging problems in environmental chemistry (Sahu et al. 2018; Ghaffar et al. 2021). Aquaculture itself is also the source of antimicrobial water contamination used in traditional aquaculture to suppress or treat bacterial diseases in aquatic animals (Vijitha et al. 2017). The development of the hydroponics business brought about the exorbitant utilization of antimicrobials to forestall or treat bacterial contaminations in fish and different creatures, especially in non-industrial nations (Bojarski et al. 2020). Antimicrobials are usually prevalent in the environment because of their misuse, overdose, and slow absorption, followed by high water solubility and resistance to

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degradation (Limbu et al. 2018). Intensive livestock farming is actually the main aspect of the environmental antimicrobial load, particularly in chickens, goats, pigs, and fish. In lower vertebrates, nearly 24.6 million pounds of antimicrobials are utilizing for non-therapeutic (prevention and growth promotion) uses, compared to only three million pounds for human medical use (Landers et al. 2012). Triclosan [5-chloro-2-(2,4-dichloro-phenoxy) phenol], a broad-spectrum antibacterial chemical, was put on a list of the ten most common terrestrial and aquatic pollutants (Dar et al. 2020). Its widespread use of wastewater treatment plants (WWTPs) of care goods, plastic, fiber, and acrylic products, and inadequate disposal (58-90%) are responsible for its sustained leakage into marine bodies worldwide (Hussain et al. 2019). Since initial successful collection from wastewater, the quantity of triclosan recycling the atmosphere in sewage sludge in the United States. This pesticide can bind to many other suspended substances in marine atmospheres, potentially endangering naval organisms and contributing to further bioaccumulation. Its presence is documented from surface waters, water body sediments, and tissues of many aquatic species, such as algae, freshwater snails, dolphins, mussels, amphibians, including fish (Gatidou et al. 2010; Dhillon et al. 2015). Because of lipophilicity, TCS has the ability for bioaccumulation in the food web and is considered to be detrimental to the environment and life even at low concentrations. Endocrine destruction involves the detrimental effect of TCS on aquatic ecosystems (Tao et al. 2019), neurotoxicity (Sahu et al. 2018), mitochondrial dysfunction (Teplova et al. 2017), oxidative stress (Gyimah et al. 2020) owing to the increased creation of various reactive oxygen species (ROS), variation of the activity of antioxidative, detoxification and impairment of immune system (Park et al. 2017; Mi et al. 2018) were reported. For almost all types of toxins on earth, aquatic bodies are the dumps, but aquatic animals, especially fish, are selected for toxicity tests because they are more sensitive to stressors than terrestrial species, like mammalian organisms (Lackner 1988; Akram et al. 2021). Extended interactions with animals with low, non-fatal, environmentally significant chemical concentrations are of major concern worldwide, with long-term exposures being the subject of regulatory scrutiny. Some clinical studies are available on the sublethal toxicity of TCS in fish, as in trout, (Capkin et al. 2017) *Cyprinus carpio*, *Ctenopharyngodon idella*, and *Cirrhinus mrigala* (Wang et al. 2019; Dar et al. 2020), *Pangasianodon hypophthalmus* (Paul et al. 2020a), Zebrafish (Falisse et al. 2017). However, Fay et al. (2018) suggested that more comparative studies are required because super-class fish are highly complex and a scientific approach to generalization would not be merely based on one or two species. Fish thrive in intimate contact with the natural world and are thus very prone to risks in the marine environment. In their blood and serum parameters, harmful changes in water content are likely to be characterized. Hematological tests are used primarily to analyze the toxicity of triclosan in marine animals, such as fish (Vijitha et al. 2017). Likewise, behavioral alternatives in fish are used in toxicological studies as an indicator of the degree and nature of toxicity (Akram et al., 2021; Sharma et al. 2019). Enzymatic tests such as acetylcholinesterase were already a commonly used biomarker for assessing neurotoxicity and have successfully shown the neurological effects of toxicants in fish (Dutta and Arends 2003; Ghaffar et al. 2021). In addition, information on histological anomalies due to histologic abnormalities are available like the toxicity of TCS in multiple tissues as like the liver, gills, kidneys, brain, and reproductive organs of animals (Gyimah et al. 2020). To assess the acute and chronic toxicity of TCS in bighead carp, a typical aquaculture species in Asia, from a food safety point of view, the above parameters were therefore chosen for the current analysis. To the best of our understanding, however, the toxicity of TCS in freshwater fish is scarce, particularly in Bighead carp. Therefore, we have tried to determine the poisonousness of TCS at sub-lethal doses in bighead carp (*Aristhicthys nobilis*) in the current report.

2. MATERIALS AND METHODS

2.1. Fish supervision and preliminary screening tests

Freshwater bighead carp, *Aristhicthys nobilis* weighted 150±170g and 8.5±10cm in length were collected from a fish farm, district Bahawalpur, Pakistan. For two weeks, fish were accustomed to research laboratory conditions with a continuous stream of de-chlorinated water. The physico-chemical properties of table water were estimated. During the trial, the water temperature varied from 26-28°C, water oxygen saturation ranged from 70 to 100%, pH from 6.5 to 7.5, which were analyzed using a uniform method. All the trial specimens were positioned in crystal aquaria according to the procedure and guidelines of the Bio-Ethics Committee of Islamia University of Bahawalpur.

2.2. Elements

Triclosan (5-chloro-2-(2, 4-dichlorophenoxy) phenol) of up to 98.5% transparency was obtained from commercial Laboratories Pvt Ltd, Punjab, Pakistan. All other chemicals were of analytical ranking and gotten from local marketable sources in Punjab.

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2.3. Experimental design and dose exposure

After fourteen days of familiarization, all fish were arbitrarily put in four equal clusters (A-D), without any corporeal aberration, vigorous and free from internal and external parasitic problems. The fish in groups (B-D) was exposed to triclosan at various concentrations. Group A fish operated as a monitoring group. Triclosan was mixed with alcohol and specimen in groups B, C and D were subjected to varying triclosan doses, such as 1000, 1500 and 2500µg/L, for 15 days. Various doses of Triclosan were used based on previous reports (Fukuhori et al. 2005; Huang et al. 2018). All fish were carefully scrutinized for any bodily and behavioral signs on a regular basis.

2.4. Hematological assessment and nuclear abnormalities of erythrocytes evaluation

Each fish was held and coated with a thin, dry filter paper and the tail end of its body was blotted with another clean, coarse filter paper. A sharp blade was used to cut the caudal peduncle (control and experimental group) of the fish with a single stroke. After discarding the first blood drop, the free-oozing blood was collected for biochemical analysis. To separate the blood serum that was used for biochemical analysis, blood was centrifuged at 5000rpm for 10min. For hematological criteria, a limited volume of blood was added to the anticoagulant (1% ethylenediamine tetra acetic acid - EDTA). During the sampling process, the blood was mixed thoroughly with the anticoagulant using a thin, blunt glass rod. The whole blood was used in both control and experimental groups for the measurement of erythrocytes, leukocyte counts and hemoglobin.

By using the Neubauer hemocytometer chamber, red blood cells (RBC) and total leukocyte count (TLC) were counted (Islam et al. 2019) at different experimental intervals on 10th of the trial. Estimation of hemoglobin concentration and hematocrit values of fish blood was described previously (Ghaffar et al. 2018), whereas nuclear abnormalities were estimated according to the method described by Hussain et al. (2019) with slight amendment. Nuclear aberrations within cell were graded among thousand cells below 100x magnifications. Erythrocyte indices were calculated as per already defined (Khan 2009).

2.5. Enzymatic assessments and histological evaluations

For various serological criteria, serum was parted from the blood of individually fish placed on the frost at various investigational periods on 10^{th} day of the experiment. A chemistry analyzer was used to evaluate different serum biochemical parameters using widely available kits (Randox Company Pvt), as well as ALT and AST, urea, creatinine and cholesterol, and triglycerides. On 10^{th} day of the experiment, 05 fish from every group were arbitrarily scored, slain and dichotomized to estimate histopathological modifications. Various organs, as the liver gills and kidneys, were extracted, pondered and stored in a 10% formaldehyde solution. Histopathological studies of multiple organs, with the gills, liver and kidneys, were executed on 10^{th} day of the research. Visceral processing done by using Hematoxylin and Eosin staining techniques for histopathological changes (Hussain et al. 2019).

2.6. Statistical analysis

In this report, the collected data is described as mean±SE. Normally, the composed data was disseminated in every group and statistical analysis was took placed by one-way analysis of variance (ANOVA), Using IBM SPSS software (Version 20). The variance in the control and treatment clusters was resolute by the Tukey test at $P<0.05$.

3. RESULTS

3.1. Nuclear abnormalities of erythrocytes

The findings of the percentile rate of various physical and nuclear alterations in of fish exposed to various concentrations of triclosan are accessible in Table 1. The fish exposed to triclosan (1500 and 2500µg/L) showed an elevated occurrence of multiple morphological defects in red blood cells. The percentile rate of erythrocytes (Pear designed), spherocytes and microcytes (Fig. 1) in fish exposed to 1500 and 2500µg/L concentrations was significantly higher at 10^{th} day of the trial relative to control groups. In RBCs of fish exposed to 1000, 1500 and 2500µg/L triclosan, the percentile rate of different changes in nucleus such as notched and blebbed micronuclei and broken nuclei was significantly increased in fish compared to the rheostat group (Table 2). Results exhibited that the percentile rate of erythrocytes with fragmented nuclear parts and erythrocyte with nuclear leftovers was knowingly higher in fish exposed to 1500 and 2500µg/L triclosan at 10^{th} day of the experiment in comparison to untreated control fish (Fig. 1).

3.2. Hematological studies and serum analysis

Consequences from the changed hematological constraints of fish blood cells showing to varying amounts of triclosan are accessible in the (Table 2). Over last 10^{th} day of the experiment, fish showing to 1500 and 2500µg/L of triclosan (groups C and D) showed a significant reduction in red blood cell count. Concentration of hemoglobin was reduced significantly in fish blood exposed to (1500 and 2500µg/L) higher concentrations of triclosan at 10^{th} day of

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experiment. Fish exposed to 1000, 1500 and 2500µg/L of triclosan exhibited that the number of differential leukocytes (neutrophilic leukocytosis) was significantly increased on 10th day of the experiment (Table 3).

The findings revealed that, relative to untreated control fish, lymphocytes, monocytes were substantially reduced in fish showed to 1500 and 2500µg/L of triclosan on 10th day of the experiment. In fish exposed to higher concentrations (1500 and 2500µg/L) of triclosan on 10th day of the trial, the number of packed blood cells was also significantly reduced. The findings of various serum biochemical parameters in fish exposed to different triclosan concentrations are presented below (Table 4). On 10th day of the trail, the quantity of serum albumin and overall serum protein substantially reduced in fish (Groups C and D) subjected to 1500 and 2500µg/L of triclosan when related to control group fish.

Table 1: Percentile rate of various morphological and nuclear alterations erythrocytes of bighead carp induced by triclosan

Parameters/days	Groups/Treatments			
	A (0.0µg/L)	B (1000µg/L)	C (1500µg/L)	D (2500µg/L)
Pear shaped erythrocyte (%)				
5	0.65±0.01	0.65±0.01	0.67±0.01	0.68±0.01
10	0.61±0.07	0.67±0.01	0.68±0.03	0.71±0.07
15	0.62±0.05	0.68±0.04	0.69±0.01	3.25±1.01*
Spherocytes (%)				
5	0.42±0.08	0.45±0.02	0.47±0.01	0.48±0.01
10	0.44±0.06	0.45±0.04	0.47±0.03	0.49±0.03
15	0.45±0.07	0.46±0.03	0.48±0.05	2.21±0.09*
Microcytes (%)				
5	0.27±0.01	0.28±0.03	0.29±0.06	0.30±0.02
10	0.26±0.03	0.27±0.05	0.29±0.03	0.32±0.03
15	0.27±0.05	0.28±0.07	2.69±0.03*	2.92±0.08*
Erythrocyte with broken nucleus (%)				
5	0.34±0.03	0.36±0.07	0.36±0.03	0.37±0.05
10	0.37±0.05	0.37±0.05	0.38±0.03	0.39±0.07
15	0.36±0.04	0.39±0.04	0.59±0.05*	0.88±0.09*
Erythrocyte with micronucleus (%)				
5	0.22±0.04	0.25±0.03	0.28±0.02	0.29±0.01
10	0.23±0.05	0.26±0.05	0.29±0.03	0.30±0.01
15	0.24±0.04	0.27±0.02	1.43±0.39*	1.97±0.08*

Values (Mean±SE) with asterisks in a row differ significantly (P<0.05) from untreated fish. Triclosan is antibacterial chemical among 10 most common terrestrial and aquatic pollutants.

Table 2: Hematological profile bighead carp exposed to different concentration of induced by triclosan

Parameters/days	Groups/Treatments			
	A (0.0µg/L)	B (1000µg/L)	C (1500µg/L)	D (2500µg/L)
Erythrocyte counts				
5	3.31±0.01	3.30±0.11	3.28±0.03	3.27±0.13
10	3.33±0.10	3.29±0.10	3.26±0.03	3.24±0.09
15	3.37±0.13	3.29±0.13	2.57±0.03*	2.44±0.19*
Hemoglobin (g/dl)				
5	10.12±0.14	10.04±0.11	9.99±0.24	9.15±0.11
10	10.15±0.11	10.02±0.19	9.79±0.33	9.70±0.13*
15	10.22±0.71	10.05±0.17	8.78±0.11*	7.11±0.01*
White blood counts				
5	16.8±0.35	17.1±0.36	18.8±0.51	19.1±0.11
10	16.9±0.31	17.4±0.48	18.8±0.79	19.8±0.06*
15	16.9±0.38	17.6±0.34	20.7±0.99*	22.6±0.61*
Pack cell volume				
5	36.4±0.31	35.5±0.53	33.2±0.38	31.3±0.43
10	37.1±0.12	34.3±0.61	31.5±0.43*	29.1±0.11*
15	36.9±0.33	34.1±0.11	29.1±1.10*	28.1±1.01*
Neutrophils (%)				
5	23.3±0.28	23.8±0.31	24.5±0.11	25.2±0.13
10	23.5±0.33	24.1±0.11	25.0±0.07	26.99±0.82*
15	22.9±0.25	24.2±0.22	28.1±0.33	29.7±0.55*

Values (Mean±SE) with asterisks in a row differ significantly (P<0.05) from untreated fish.

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Table 3: Different serum biochemistry parameters of bighead carp exposed to different concentrations of triclosan

Parameters/days	Groups/Treatments			
	A (0.0µg/L)	B (1000µg/L)	C (1500µg/L)	D (2500µg/L)
Total proteins (mg/dL)				
5	3.34±0.03	3.33±0.02	3.31±0.02	3.18±0.05
10	3.35±0.04	3.24±0.02	3.11±0.05	3.01±0.01
15	3.36±0.02	3.25±0.04	2.81±0.10*	2.25±0.05*
Aspartate aminotransferase (U/L)				
5	11.7±0.13	12.1±0.11	13.15±0.16	14.11±0.13
10	11.4±0.15	12.6±0.12	14.22±0.22	14.55±0.33
15	11.5±0.11	12.8±0.21	16.35±0.33*	17.11±0.44*
Alanine aminotransferase (U/L)				
5	18.87±0.17	19.12±0.23	20.11±0.12	21.4±0.13
10	18.93±0.33	20.11±0.17	21.9±0.13	23.1±0.05
15	19.65±0.26	21.1±0.11	25.5±0.78*	26.2±0.17*
Urea (mg/dL)				
5	7.11±0.03	7.14±0.11	8.19±0.22	8.33±0.11
10	8.01±0.02	8.13±0.19	9.33±0.33	9.752±0.31
15	7.09±0.09	8.14±0.03	9.99±0.31*	10.5±0.22*
Creatinine (mg/dL)				
5	1.18±0.03	1.25±0.02	1.29±0.01	1.30±0.03
10	1.19±0.08	1.27±0.10	1.30±0.03	1.32±0.01
15	1.18±0.09	1.29±0.09	1.65±0.01*	1.74±0.05*
Cholesterol (mg/dL)				
5	132.4±2.11	135.3±1.22	141.3±1.37	143.1±1.9
10	135.1±2.15	137.7±2.13	142.1±1.22	144.1±1.15
15	137.3±2.01	138.6±2.19	149.1±3.11*	153.1±2.23*
Triglycerides (mg/dL)				
5	155.4±5.11	156.2±3.55	159.5±3.13	160.3±2.11
10	157.3±4.15	158.5±2.85	161.1±2.91	162.3±1.55
15	156.5±3.15	159.5±1.89	169.1±3.33*	172.7±2.29*

Values (Mean±SE) with asterisks in a row differ significantly (P<0.05) from untreated fish

Table 4: Severity of various microscopic alterations in different visceral tissues of bighead carp treated with triclosan

Organs/Histopathological lesions	Groups/Treatments		
	B (1000µg/L)	C (1500µg/L)	D (2500µg /L)
Liver			
Pyknosis	+	++	+++
Vacuolar degeneration	++	++	++++
Degeneration of hepatocyte	++	++	+++
Hemorrhages	+	+++	++++
Kidneys			
Deterioration of glomerulus	+	++	+++
Necrosis of tubular cells	+	++	++++
Congestion	+	+++	+++
Increased Bowman's space	+	++	++++
Ceroid formation	+	++	+++
Brain			
Necrosis of neurons	+	++	+++
Congestion of neural cells	-	++	++
Intracellular oedema	+	++	++
Cytoplasmic vacuolization	++	+++	+++
Atrophy of neuron	+	++	+++
Gills			
Necrosis of lamellar epithelial cells	+	++	+++
Disruption of primary lamellae	+	+++	++++
Congestion	+	++	+++
Congested cartilaginous core	+	++	++
Lamellar atrophy	++	++	+++
Lamellar disorganization	++	+++	+++

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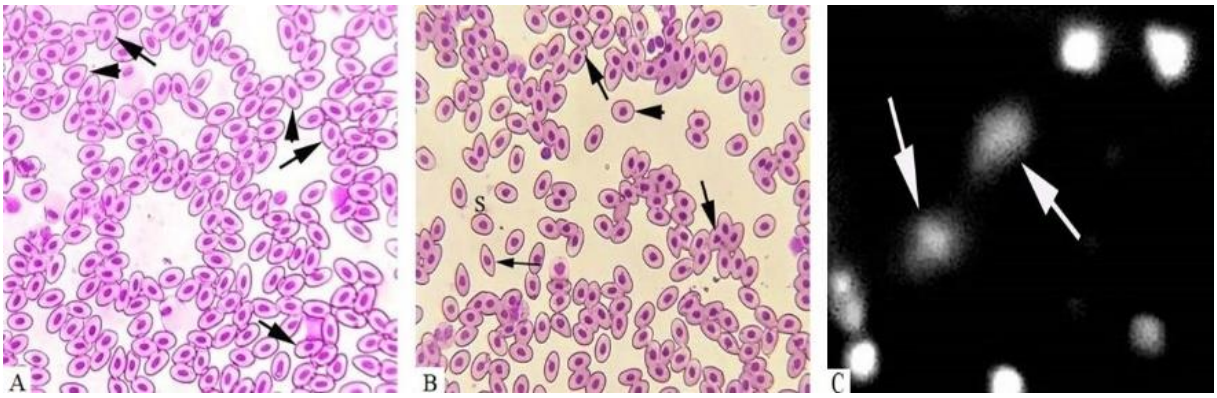

Fig. 1: Photomicrograph showing different morphological and nuclear alterations along with DNA damage in bighead carp treated with triclosan (1500 and 2500µg/L). A) Erythrocyte with condensed nuclei (arrows) and pear shape erythrocytes (arrow heads), B) erythrocytes with micronuclei (thick arrows), spindle shape erythrocytes (thin arrows) and spherical erythrocyte (arrowhead) and C) comet assay showing DNA damage of lymphocytes (arrows). Geimsa Stain. 1000X.

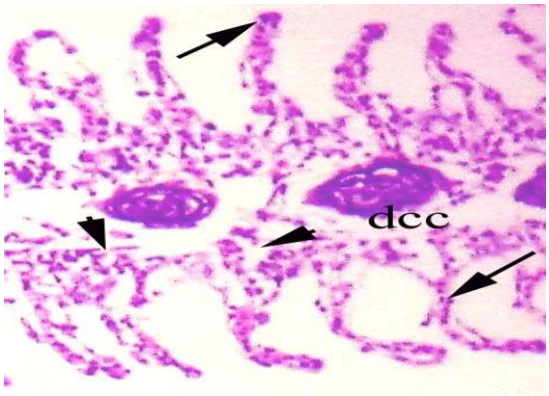

Fig. 2: Photomicrograph of gills tissues of fish treated with Triclosan (2500µg/L) showing disorganization of secondary lamellae (arrow heads), necrosed lamellar pillar cells (arrows) and disorganization of cartilaginous core (dcc). H & E. 400X.

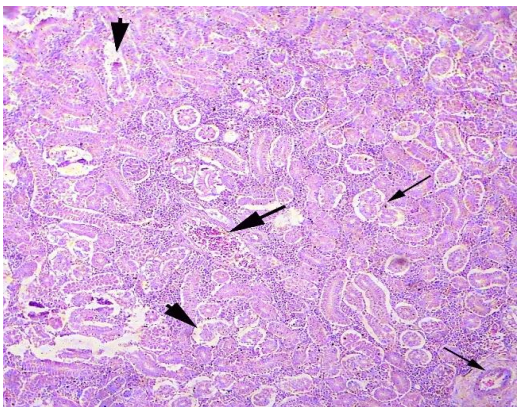

Fig. 3: Photomicrograph of kidneys of fish treated with Triclosan (2500µg/L) showing necrosis of renal tubules (thin arrows), congestion (thick arrows) and degeneration of tubules (arrow heads). H & E.200X.

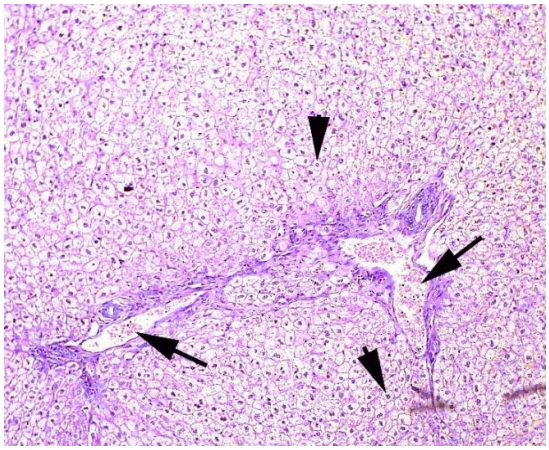

Fig. 4: Photomicrograph of liver of fish treated with Triclosan (2500µg/L) showing congestion (arrows) and necrosis (arrow heads). H & E. 200X.

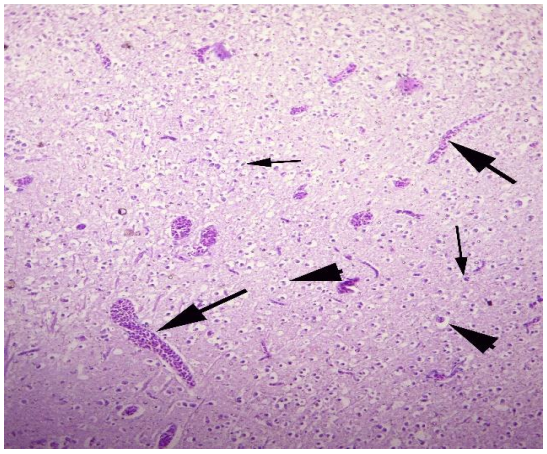

Fig. 5: Photomicrograph of brain of fish treated with Triclosan (2500µg/L) showing various microscopic lesions congestion (arrows), necrosis of neurons (thin arrows) and atrophy of neuron (arrow heads). H & E.200X.

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The amount of serum alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST) and serum alkaline phosphatase (ALP) suggestively raised in fish liver tissue at higher triclosan concentrations (1500 and 2500µg/L) on 10th day of the experiment (Table 3). Serum triglycerides in fish treated to 1000, 1500 and 2500µg/L were significantly raised on last 10th day of the trail. The amount of urea and creatinine significantly enhanced compared to control fish kidneys, exposed to 1500 and 2500 µg/L of triclosan at 10th day of the experiment. In treated groups (B-D) fish exposed to 1000, 1500 and 2500µg/L the glucose level significantly increased on 10th day of the study. Cholesterol and lactate dehydrogenase significantly raised in fish exposed to 1500 and 2500µg/L of triclosan relative to control fish (Table 3).

3.3. Histopathology

Histopathological alterations in various fish tissues exposed to different doses of triclosan are shown in Table 4. Severe histopathological abnormalities in gill tissues of fish such as disorganization of lamellae, necrosed lamellar pillar (Fig. 2), primary lamellar disintegration, curling and fusion of secondary lamellae, extreme congestion and worsening of cartilaginous cores were observed in groups C and D of trail. Curling, uplifting of epithelial cells of secondary lamellae were generally reported in C and D clusters of 10th day trail. In the kidneys of chemical treated groups (B-D), there were seen mild to moderate histopathological changes like pyknosis, deterioration, congestion, elevation in Bowman's capsule, edema, tubular epithelial disintegration, melano-macrophage accumulation and luminal atrophy were observed (Fig. 3). Major histopathological changes were appeared in the kidneys of fish in B group at 10th day of experiment, such as glomerular degradation, necrosis of tubular cells. Medium to extreme histopathological changes in the liver tissues of fish, namely congestion (Fig. 4), cirrhosis, hemorrhage, karyorrhexis, karyolysis, hypertrophy of nucleus and eccentric nucleus, fragmented vacuole fragmentation was noted on 10th day of the experiment. Various histopathological liver problems in fish (*Aristhicthys nobilis*) exposed to doses of 1500 and 2500µg/L showed vacuolar disintegration, karyorrhexis, karyolysis on 10th day of the research investigation. Results on brain histopathology exhibited different microscopic lesions like congestion, necrosis and atrophy of neuron (Fig. 5) in triclosan treated fish.

4. DISCUSSION

Triclosan is a popular ingredient in a variety of everyday cleaning products, including hand soaps, dish and laundry soaps, and personal care products like toothpaste and mouthwash, and can be found in institutional, clinical, commercial, and residential settings. These substances can be flushed down the drain, survive wastewater treatment, and end up in rivers. Because triclosan is lipophilic, it is rapidly absorbed and bioaccumulated in fatty tissues, particularly in aquatic species. Fish have been shown to collect substantial amounts of Triclosan metabolites, according to studies. Due to triclosan, a significantly higher frequency of various morphological ailments in red blood cells of bighead carp was observed, including microcytes, leptocytes, spherocytes, pear-shaped and various nuclear aberrations such as lobed, blebbed and notched nucleus, erythrocyte with micronucleus, vacuolated nucleus, nuclear ruminants in red blood cells. Previously, similar results like formation of micronucleus along with lobed, blebbed, notched, irregular and apoptotic nucleus in fish *Oreochromis niloticus* (Vijitha et al. 2017) exposed to sublethal dose of triclosan was reported. Moreover, similar reports on erythrocytic abnormalities in American bullfrog, *lithobates catesbeianus* (Curi et al. 2019), Rain bow trout *Labio rohita* (Jindal and Verma 2015) and rats and Hamsters (Rodricks et al. 2010; Environment and Climate Change Canada Health Canada, 2016), were observed in previous reports. The blood of fish subjected to various dosages of triclosan was tested for reduced haemoglobin content, monocytes, lymphocytes, and pack cell volume. Due to fast oxidation of haemoglobin, hemolysis, and erythrocyte deterioration, parameter values are reduced (Ghaffar et al. 2016; Gul et al. 2017). Rise in the number of neutrophils may rise also be attributed to immunological reactions shown by tissue grievance in exposed bighead carp. In one of the previous reports, decreased RBC and hemoglobin and this was due to hemolyzing capacity of triclosan and rapid oxidation of hemoglobin to methemoglobin. However, WBC content was significantly increased owing to leucocyte mobilization, as the defensive mechanism of fish *Oreochromis niloticus* (Vijitha et al. 2017) exposed to different concentration of triclosan. Moreover, similar reports like significant decrease in hematological parameters related to complete blood counts except for TLC, mean corpuscular hemoglobin concentration (MCHC) and red cell distribution width (RDW) were studied in *Pangasianodon* fish (Sahu et al. 2018; Paul et al. 2020b) exposed to different doses of triclosan. However, significant ($P<0.05$) decrease in hemoglobin (Hb) content, RBC, hematocrit and mean corpuscular volume (MCV) in triclosan exposed fish, which indicates the disruptive action of triclosan on erythropoitic tissues, caused shrinkage in red blood cells and making the erythrocytes more brittle and porous. Whereas significant ($P<0.05$) increase in TLC, mean corpuscular hemoglobin (MCH) and MCHC indicates the direct or indirect responses of structural damage in RBC membranes resulting in hemolysis in fish *Labio rohita* exposed to different doses of triclosan (Geetha and Priya 2020).

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In the current study, serum biochemical markers (ALT, AST, and ALP) significantly increased in response to triclosan-induced stress. Serum biochemical indicators such as serum albumin amount and serum total protein were shown to be lower in fish blood in the current study. However, stress circumstances in treated fish resulted in elevated glucose, cholesterol, and lactate dehydrogenase levels were studied. Serum creatinine and uric acid are very essential factors for muscle and purine metabolism of kidney performance (Akram et al. 2021). The quantity of urea and creatinine in the liver and kidney were also enhanced, reflecting disruption of filtration mechanisms and damage to the kidneys and liver tissues of fish exposed to triclosan in the current experiment. Previously, change in serum lysozyme activity (enzymatic activity), total immunoglobulin in serum, ceruloplasmin level, serum total protein, albumin and globulin quantity in fish *Pangasianodon hypophthalmus* (Bera et al. 2020) was evident in TCS exposed groups in comparison to control during the experimental periods. However, in *Cyprinus carpio* (Tasneem and Yasmeen 2020), total glycogen, SDH and LDH significantly reduced as the days of sub-lethal exposure of triclosan increased in fish was reported. Similar reports were observed in *Channa punctatus* (Agrahari et al. 2007), *Labeo rohita* (Ghaffar et al. 2021), *Rhamdia quelen* (Banaee and Ahmadi 2011), *Oreochromis niloticus* (Banaee and Ahmadi 2011), exposed to toxicant. In the present research work, Histopathological lesions in bighead carp liver tissue were congestion, cytoplasmic volume reduction, expanded sinusoidal space, hepatocyte karyolysis, and necrosed tissues of liver subjected to higher triclosan concentrations. Previously, histological alterations in liver of tadpole larvae of frog Bufo gargarizans (Chai et al. 2017) exposed to different doses of triclosan was reported. Previously, triclosan-exposed necrotic hepatocytes in the liver (Capkin et al. 2017). Histopathological alterations in the liver of *Catla catla* (Hemalatha et al. 2019) fish subjected to various triclosan concentrations were also investigated. Similar findings, such as swelling, epithelial elevating, and lamellar fusion, were also observed, along with hepatocyte vacuolation, nuclear pyknosis, and hepatocyte vacuolation, nuclear pyknosis, hepatocytic degeneration, sinusoid extension, and hemorrhage in *Oncorhynchus mykiss* (Rodrigues et al. 2019), *Barbonymus gonionotus* (Islam et al. 2015) were reported in previous studies. In current trial, microscopic lesions such as edema, cirrhosis, glomerular deterioration, Bowman's space, tubule congestion and lumenal atrophy were also seen in the kidneys of treated fishes. Previously, Regeneration of damaged epithelial cells, edema, glomerular degeneration was observed in *Oreochromis nilotica (*Augusto et al. 1996), *Opsanus tau* (Reimschuessel et al. 1996), *Carassius auratus* (Reimschuessel and Williams 1995), exposed to toxicants. Presently, Because the gills have direct contact with water pollutants and are the first organ into which contaminants penetrate, literature has shown that the development of histopathological changes in fish gills is a very effective tool for measuring the impact of various pollutants in the freshwater environment. Gills are those essential organs that serve as a gas exchange medium, boundary between water and fish, balancer of ionic compounds and are responsible for the process of osmoregulation (Ghaffar et al. 2018). In the current research, histopathological lesions in fish gills include atrophy, fusion, uplifting, obstruction, and primary secondary lamellae disorganization. Previously, related histological alterations were observed in zebrafish (O'Brown et al. 2018) exposed to sublethal doses of toxicant. The literature contains limited information on the effect of antibacterial chemical on the biochemical analysis in profile of freshwater fish of their use as biomarkers of contamination (Hussain et al. 2019). The results of this research have shown that triclosan contaminants are likely to have adversely affected the fish. Therefore, to verify these findings, more exploratory work needs to be done.

Conclusion: The findings showed that triclosan changes the hematological, histopathological, and biochemical parameters of the fish A. nobilis at sublethal concentrations, and that these parameters can be used to detect triclosan adverse effects in aquatic environments and to determine the physiological condition of fish. As a result, histopathological examinations are carried out to determine the extent of damage to key organs such as the liver, kidneys, and gills. The introduction of such chemicals into rivers, on the other hand, should be limited, despite the fact that carps (A. nobilis) are natural occupants of freshwater habitats and are a sought-after food species in nations like Pakistan. However, more research work is required to verify these findings.

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TRANSFORMATION AND EXPRESSION OF GENE IN MICROALGAE: FROM TOOLS TO APPLICATIONS

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ABSTRACT

Microalgae provide an excellent platform for foreign genes expression. Chloroplast and Nucleus based transformation of transgenes in algae is possible after construct designing (including different markers and promoters) and codon optimization. Different and effective promoters and reporter genes are selected for better recombination. Chloroplast mediated transformation is much more effective than nuclear-based transformation for transgene analysis owing to the higher copy number of chloroplast in the unit area. Microalgae-based products are an excellent target for producing a wide variety of bio-products such as vaccines, antibiotics, and drugs against different diseases. These alternative approaches facilitate enhanced biodiesel production through the fast growth of microalgae in a heterotrophic environment. Also, low risks are attributed owing to their nonhuman consumption property. Biosafety and risk assessment should be carried out before releasing genetically modified microalgae into the environment, considering ethical research code. Microalgae provide cheap, efficient, and high-yielding alternatives to plants. The review emphasizes a microalgae-based expression approach with key steps involved in detail.

***Keywords*:** Microalgae, Transformation, Expression, Codon optimization, Biodiesel, Vaccines

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1. INTRODUCTION

Owing to the exponential growth of global population, demand for a number of key industrial products is on the rise. Traditional production systems' costs are on rise due to high mandate and low product yields, which is a strong reason to design solutions for low-cost production (Siddiqui et al. 2020). Microalgae are a varied group of prokaryotic and eukaryotic organisms that play an important ecological role. They are responsible for around half of all global organic carbon fixations (Leon and Fernandez 2007). Photosynthetic microalgae are of vast interest these days as they provide a sustainable approach for the production of large number of products in the field of pharmaceutical, nutrition and biofuels, if genetically engineered. A number of microalgae species have been assigned GRAS (Generally Recognized as Safe) status from US and FDA for human consumption (Torres-Tiji et al. 2020; Sproles et al. 2021). Algal species can be used for producing recombinant proteins by inserting desired gene into the genome of microalgae (Shirakawa et al. 2021). Microalgae give a production stage to the outflow of foreign genes because of their fast development under photoautotrophic conditions and their minimal effort of support, when contrasted with land plants, mammalian cells, yeast and bacteria. Likewise, numerous algal species have created novel metabolic tracks that deliver mixes of commercial esteem (Siddiqui et al. 2020). Microalgae may produce a wide range of high-value bioactive chemicals, including carbohydrates, proteins, lipids, essential fatty acids, pigments, vitamins and antioxidants, among other thing (Udayan et al. 2021). Microalgae are being regarded as prospective bioremediators of urban and agro-industrial effluents, allowing the integration of bio refineries, the utilization of gaseous effluents and the development of added value biomass in this context (dos Santos et al. 2021). Concern about looking young and beautiful has grown in the previous decade, prompting many customers to use anti-aging cosmetics on a daily basis. Cosmetic companies have recently begun to investigate the use of chemicals isolated from microalgae and cyanobacteria in new formulations (Morocho-Jácome et al. 2020). The model diatom

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algae species *Phaeodactylum tricornutum* is an appealing option for synthetic biology applications (Scaife and Smith 2016; Brasil et al. 2017). Because of its inherent proclivity for lipid storage, *P. tricornutum* is an attractive choice for biofuel generation. The nuclear, mitochondrial and plastid genomes of this algae species were sequenced due to industrial and academic interest (Bowler et al. 2008; Yongmanitchai and Ward 1991; Cochrane et al. 2020). The chloroplast of microalgae like *Chlamydomonas reinhardtii* is an appealing chassis for producing novel recombinant proteins and metabolites using light. Over 100 different proteins have been successfully produced using methods for introducing and expressing transgenes in the chloroplast genome of *C. reinhardtii* (Larrea-Alvarez and Purton 2020). In the biotechnologically important alga *Picochlorumceleri*, Cas9-guide RNA ribonucleoprotein (RNP) complexes were employed to efficiently create insertions in both alleles of two targeted genes (nitrate reductase (Fig. 1) and carotenoid isomerase) (Krishnan et al. 2020). Transformation approaches are a critical component of functional genomics research. Microalgae genetic transformation is quickly becoming a valuable biotechnological tool to achieve all the objectives discussed above (Ortiz-Matamoros et al. 2018). The microalgal cell has three membrane-bound organelles: nuclei, chloroplasts, and mitochondria. Despite the fact that most of the preceding instances employ nuclear transformation, nuclear expression has the disadvantage of being susceptible to epigenetic effects and random insertion, which can result in fluctuating transgene expression Chloroplast engineering, on the other hand, has numerous major advantages, including the absence of epigenetic gene silencing, selective transgenic location in the genomes, and relatively high expression levels (Leister 2003; Zhang et al. 2017; Lu et al. 2021).

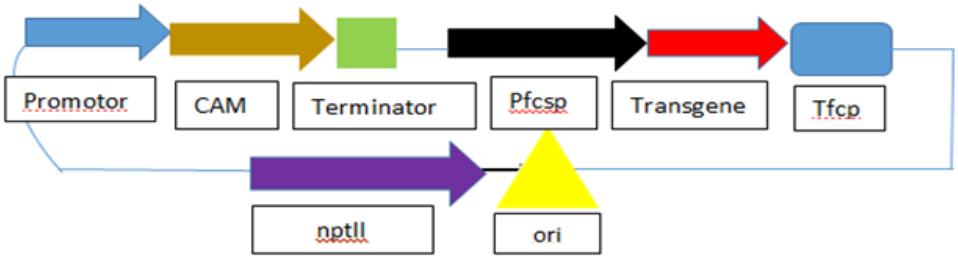

Fig. 1: Systemic view of possible vector construct in microalgae. Annotations of the Plasmid Map: Promoter and Terminator (nitrate reductase) from microalgae, CAM is Reporter gene of chloramphenicol acetyltransferase, PfcpCis Promoter of fcpC gene from microalgae TfcpA: Terminator of fcpA geneoriis pBR322 site for replication. nptIIis neomycin phosphor-transferase gene extracted from Tn5 transposon, it is a reporter gene. AmpR can also be used.

1.1. Nuclear Transformation

For the transformation of microalgae, various transformation procedures have been established. Despite improved capabilities and diverse ways to producing a variety of bioproducts from microalgae, there are technological problems and constraints that must be overcome before algal biotechnology can be commercialized (Gimpel et al. 2015). By various physical/chemical techniques, algal genetic transformation processes create temporal permeabilization of the cell membrane/cell wall, allowing DNA molecules to enter the cell. Thus, with or without external aid, DNA enters the nucleus through the nuclear membrane and integrates into the genome (Hallmann 2007; Rathod et al. 2017). In Chlamydomonas cell wall contains low level of chitin or cellulose so protoplast is generated by incubating with autolysins which are named as hydroxyl-proline-specific protease (Jaenicke et al. 1987; Patil et al. 2020). Biolisticdelivery includes DNA coated gold or tungsten which is delivered with high velocity through particle delivery system by transcending the cell wall which is a physical barrier (Brown et al. 2020).). The protoplast of microalgae is stirred in the presence of glass beads, polyethylene glycol (PEG), and foreign DNA in the Glass Beads technique (Zhang et al. 2020). Electroporation another method for introducing DNA is by applying electric impulses (Table 1). This method is utilized to transform to protoplast and thin wall algal cell. This method was used to transform *C. reinhardtii* (Ng et al. 2020).). *Agrobacterium*-based transformation of microalgae like in land plants Agrobacterium tumefaciens is used to transfer nucleus using pCAMBIA transformation vector. Similarly, it is also used to transfer nucleus in various microalgae like Chlamydomonas (Kathiresan et al. 2009; Dehghani et al. 2017).

1.2. Chloroplast Transformation

Chloroplast plays an important role in the reduction of atmospheric carbon dioxide by utilizing it in the production of most abundant protein of the Earth, RuBisCO. In recent decades, genetic modification of chloroplasts

Sadaqat S, Usmaan M, Mizna, Shakoor S, Hameed R, Ajmal S, Yaqoob A, Muhammad M, Babar M, Ishaq A, Irfan M, Ain NU, Awais M, Bhutta MS, Raouf A, Imtiaz M, Shahid N, Latif A, Yasmeen A, Azam S, Samiullah TR, Rao AQ and Shahid AA, 2022. Transformation and expression of gene in microalgae: From tools to applications. Agrobiological Records, 7: 29-39. https://doi.org/10.47278/journal.abr/2021.012

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has been in practice for producing therapeutic proteins and enzymes used in daily life (Ruhlman et al. 2010; Daniell et al. 2021). High levels of desired proteins expressed in chloroplast can be obtained due to high copy number of that organelle. It has a full genetic system with 100–200 genes, the majority of which are connected to photosynthetic light reactions or transcription and translation processes (Green 2011; Bo et al. 2020). Multiple genes can be produced as polycistronic transcription units in the chloroplast concurrently under the control of a single promoter, allowing for the coordinated synthesis of enzymes from certain metabolic pathways or subunits of a protein complex (Kwon et al. 2018). The main stable framework for the chloroplast of *C. reinhardtii* was built up utilizing biolistic delivery. By homologous recombination of the transgenic marker into the goal mutant strain, the outside DNA was supposed to rescue three mutants of the chloroplast atpB quality and restore photosynthetic activity (Boynton et al. 1988; Kwon et al. 2018).

1.3. Markers for the Chloroplast Transformation

Different markers are being used for chloroplast transformation on the basis of their growth, metabolic activity and many other factors listed below.

1.4. Photoautotrophic Growth

Chlamydomonas has the advantage of being able to grow in non-photosynthetic environments while utilizing acetate as a carbon source. Thus, non-photosynthetic mutants were used as recipient strains and retrieval of their photosynthetic action was used as a reporter system to restore expression of the mutated atpB gene, which codes for ATP synthase, and the tscA gene, which codes for a small RNA involved in trans-splicing of the psaA transcript (Goldschmidt-Clermont 1991; Day and Goldschmidt-Clermont 2011). When a foreign gene is added into the plastid genome, native expression cues such as a chloroplast promoter, a 5′UTR with translation initiation signals, and a 3′UTR are required. A variety of chloroplast expression signals have been used in Chlamydomonas, including those from atpA, psaA, psbA, and psbD (Day and Goldschmidt-Clermont 2011; Michelet et al. 2011).

1.5. Metabolic Enzymes

Plastid transformation selectable markers are mainly based on bacteria genes. The expression of bacterial gene ptxD in the chloroplast that encodes an NAD (P)-dependent phosphite oxidoreductase to allow utilization of phosphite as a source of phosphorus (P) (Sandoval-Vargas et al. 2019). The approach aimed that most organisms cannot use phosphite, therefore, growing the transgenic microalga in phosphite provides a selective advantage over competing species (Dyo and Purton 2018).

1.6. Resistance to Antibiotics

Plastid's translation machinery has preserved prokaryotic characteristics, so mutations in ribosomal proteins or rRNA can provide resistance to antibiotics like spectinomycin, streptomycin, and erythromycin. These serve as indicators of chloroplast transformation. Another option is to employ marker genes that code for enzymes that alter and inactivate antibiotics chemically (Day and Goldschmidt-Clermont 2011). Resistivity against the spectinomycin was given by presenting the bacterial-determined aadA quality in the genome of chloroplast, which encodes for aminoglycoside 3′ adenyl- transferase (Tabatabaei et al. 2017). When flanked by suitable chloroplast expression signals (promoter and UTRs), this bacterial gene confers significant levels of antibiotic resistance in Chlamydomonas (Bock 2015).

1.7. Resistance to Herbicides

The herbicide named as sulfometuron methyl (SMM) that restrains development of microscopic organisms, yeast, green growth and plants is generally utilized as a choice marker for plants and green growth. The objective of SMM includes quality that encodes aceto-hydroxyacid synthase (AHAS), a compound required in the biosynthesis of expanded amino acids (Grundman et al. 2012). It is observed to be an optimum marker for chloroplast alteration after the genome of chloroplast encodes AHAS in porphyridium sp. (red microalgae) resulted in the change of chloroplast genome (Doron et al. 2016).

1.8. Regulatory Elements: promoters, UTRs, and codon optimization

Transformation efficiency is very high if there is perfect homology of plastid DNA flanking sequences. Identification of promoters, 5′-UTRs, 3′-UTRs and insertion sites play a critical role in the successful transformation. Complete chloroplast genome sequences are required for integration of the transgene at exact site via homologous recombination and to identify endogenous regulatory sequences for perfect transgene expression

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(Grevich and Daniell 2005; Ruhlman et al. 2010). In green algae, diatoms, and cyanobacteria, novel endogenous promoters with both constitutive and inducible activities have recently been discovered. Different promoters like rbcL (ribulosebisphosphate carboxylase/oxygenase), psbA (D1 protein of the photosystem II response focus) and atpA (Y subunit of ATP synthase was used to optimize the activity of the promoters and atpA was resulted in the highest regulatory activity (Barrera et al. 2014).

Table 1: This table shows different pharmaceutically important recombinant proteins in different micro algae along with other important information

Microalgae	Method of transformation	Gene expressed	Promoters	Selective marker gene	Products	Expression location
D. salina	Electroporation	HBsAg	ubiquitin	Chloraamphenicol resistant gene	HBsAg	Nucleus
D. tertiolecta	Particle's bombardment	Xylanase/ alpha-glactosidase	psbD	Erythmycin resistant gene	Xylanase/alpha glactosidase	Chloroplast
Porphyridium sp.	Particle's bombardment	AHAS	Endogenous AHAS promotor	AHAS	Acetohydroxy acid synthase	Chloroplast
S. microadriaticum	Agrobacterium	GUS	CaMV35S	hpt gene	ß-glucoronidase	Nucleus
C. reinhardtii	Particle bombardment	E2	atp A	aad A	Swine fever virus structure protein	Chloroplast

Source: Chew et al. (2017); Yan et al. (2016)

1.9. Inducible Expression in the Chloroplast

For a variety of reasons, an inducible chloroplast gene expression system could be highly valuable. Primarily, it may be used to figure out how important chloroplast genes are for cell development and survival. Second, it may be quite useful for expressing proteins that are harmful to algal cells. Third, it would allow for the progressive depletion of photosynthetic complexes, making it possible to create new photosynthetic complexes (Ramundo et al. 2014). NAC2 is an important component used for the stability of psbD mRNA which binds a distinctive target site in the psbD 5′UTR and regulates the expression of a foreign gene which is under the control of psbD 5′UTR (León et al. 2008).

1.10. Promoter Selection and Codon Optimization

Genetic engineering aims to synthesize a plasmid vector comprising components necessary for gene expressional studies. A classic vector creation includes the management of selection marker expression cassettes for the selection of bacterial plasmids or genetic transfer into the host. An extra gene directed expression cassette is present for expression system (Wang et al. 2017). For that purpose, inducible promoter is used to restrict the selection marker expression in transgenic host (Trassaert et al. 2017). So many different promoters which are used for transformation of plants can be used for transformation of microalgae for instance 35S Cauliflower Mosaic Virus (CaMV) promoter and promoter of Agrobacterium p1-2, both were used for reported GUS gene expression in dinoflagellates species, Amphidinium as well as Symbiodinium (Grunennvaldt et al. 2015). Whereas RBCS2 promoter derived from Chlamydomonas reinhardtii (unicellular green algae) displayed high efficiency in contrast to 35S promoter in recombinant Dunaliellasalina (Scranton et al. 2016). Chlorella genetic reformation is relatively slow. Ubiquitin gene promoter performed satisfactory role in expression of mature Neutrophil Peptide-1 (NP-1) of rabbit in Chlorella (Run et al. 2016). While in other research *C. ellipsoidea* was transferred to a vector also having gene for flounder Growth Hormone (fGH) controlled by promoter (35S) as well as phleomycin resistance gene controlled by Chlamydomonas promoter (RBCS2) (Yang et al. 2016).

Effective expression of subject genes in chloroplasts of microalgae is usually best acquired by the usage of regulatory components, taken from genes which express abundantly. For this persistence, promoters involved in the expression of ribulosebisphosphate carboxylase or oxygenase subunits (rbcL), photosystem II reaction centre D1protein (psbA), and ATP synthase Y subunit were utilised. The maximum expression of GUS reporter gene was seen under the control of atpA promoter as well as the 5' UTRs (Doron et al. 2016). Moreover, in another research, atpA in addition to psbD promoters as well as their 5′UTR were observed to manipulate utmost expression of Green fluorescent protein (GFP), a reporter gene, when analyzed it to the promoter of rbcL as well as psbA with 5′UTR. The occurrence of 3′UTRs taken from various genes was required but they had a negligible effect on the gathering

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of transcripts as well as recombinant proteins (Rosenberg et al. 2015). In later studies, the Gold Schmidt Clermont scientists confirmed the fact that the psaA-exon1 along with its promoter as well as 5′UTRs enhances the expression of protein of interest in chloroplast. They also explained that the change in expression rate in different expression systems is also due to the location and number of events of recombination (Michelet et al. 2011). Additional developments amplified the rate of expression in *C. reinhardtii* chloroplast up to 20% of total protein content by concentrating on codon optimization as well as ATP dependent proteases inhibition (Freudenberg et al. 2021). The toxicity levels of transgene could result in adverse effects on gene expression as genotype modification associated with random insertion of gene of interest in nuclear DNA, thus many screenings analysis is needed to separate highest expression level clones. In short, the promoter, UTRs or expression techniques are not the only factors affecting rate of expression. Random integration in nucleus as well as in chloroplast integration site overall affect the expression of transgene (Ahmad et al. 2021).

Even though all of the elements required for effective transcription and translation (promoters, introns, and other regulatory regions) have been included in the chimeric gene design, exogenous gene expression can be minimal or non-existent. Furthermore, expression of the exogenous gene may be reduced if transgenic algal clones are not maintained under selection circumstances (León-Bañares et al. 2004).

1.11. Applications of Microalgae

Microalgae are unicellular photosynthetic organisms that have recently piqued interest due to their potential applications in food, nutraceuticals, medicines, animal feed, cosmetics, and bio fertilizers. Microalgae include a number of high-value bioactive chemicals that have potential health advantages and can be utilized to prevent and treat a variety of diseases. Microalgae have different uses in biotechnology including the biodiesel production, increased lipid content in soybean and pharmaceuticals. They are explained in detail below.

1.12. Pharmaceuticals

Most of the pharmaceutically important products have been made in microalgae especially in *C. reinhardtii* using different genetic engineering techniques. *C. reinhardtii* is an excellent bioreactor for the manufacture of a wide range of pharmaceutically significant proteins, such as vaccines, antibodies, therapeutic proteins, and so on (El-Ayouty et al. 2019).

1.13. Subunit Vaccine

Vaccines have been developed to combat a variety of viral and non-viral diseases. Chlamydomonas chloroplasts can create E2 protein, which contains the antigen for protein against the swine flu virus. This E2 transformed algae when introduced in mice via subcutaneous injections, a significant increase in serum antibodies against the virus was seen (Shamriz and Ofoghi 2016). Microalgae-made vaccine prototypes include four against *Plasmodium falciparum* (malaria) and one against *Staphylococcus aureus*, *Escherichia coli*, *Human papillomavirus*, *Hepatitis B virus*, *Human immunodeficiency virus*, *Ebola virus*, *Influenza virus*, and *Zika virus*. Microalgae-made vaccines against important bacterial (i. e. tuberculosis, anthrax), fungal (i.e. candidiasis), or parasitic (i.e. Chagas) diseases, among others (neglected tropical diseases), are an open field to research and development (R&D) (Hotez 2018). Importantly, preclinical evaluations have been carried out, further clinical studies are the next step to be performed. In those reports, immunizations have been carried out by intraperitoneal, subcutaneous, and intramuscular routes; remarkably, the oral route for immunization has mainly been used (Fig. 2). This fact supports that the first ideal goal of microalgae-vaccine technology is being promoted microalgae used as subunit vaccine production host and delivery vehicle (Ramos-Vega et al. 2021). For the treatment of foot and mouth disease vaccine in *C. reinhardtii*, the VP1 protein was fused with cholera toxin B subunit, which is an important adjuvant and thus the recombinant protein showed the binding affinity towards GM1 gangliosidase (Ramos-Vega et al. 2021). The D. salina genome was effectively converted with the hepatitis B virus surface antigen gene (Soria-Guerra et al. 2014). The viral envelop protein gene VP28 for White Spot Syndrome Virus (WSSV) was successfully transformed into the nuclear genome of *C. reinhardtii* resulting in recombinant *C. reinhardtii* providing a suitable candidate for oral vaccine against this WSSV (Lanh et al. 2021).

1.14. Antibodies Production

Microalgae chloroplasts developed the first monoclonal antibody against herpes simplex virus glycoprotein D. Defensins are the small antibodies like molecules produced against many pathogens. These are the small cationic peptides having same function as antibody. Mature rabbit neutrophil peptide called alpha defensin has a vast range of anti-microbial properties against virus, bacteria, pathogenic fungi etc. have been transformed into a microalgae called *C. ellipsoidea*. Growth hormone can also be expressed in *C. ellipsoidea*. *P. tricornutum* was used for

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expression of human CL4 monoclonal antibody (mAb) and using hepatitis B surface antigens evaluation was done (Hempel et al. 2011). *P. tricorntum* can show expression of recombinant monoclonal antibody against Marburg virus. *C. reinhardtii* has also been used to counter glycoprotein of herpes simplex virus by producing immunoglobulin A (Ig A) monoclonal antibody using microalgae, which lack machinery for N-glycosylation (Mayfield et al. 2003). Genetic or chemical conjugation of eukaryotic toxins and antibody fragments has been used for the production of immunotoxins (Yusibov et al. 2016). These toxins conjugated antibodies reach target cells and release toxins. These toxins disturb translational mechanism of cells and result in apoptosis of these target cells. Single chain variable fragment against CD22 antigen B-cell surface receptor of exotoxin A from *Pseudomonas aeruginosa* has been produced using *C. reinhardtii* chloroplast (Bogner et al. 2010). Erythropoietin from humans had been expressed in *C. reinhardtii* which had the activity of hsp 70 A/rbcS2 (Eichler-Stahlberg et al. 2009). 100 ng HGH from cell extract of modified *C.reinhardtii* when treated to Nb2-11 rat lymphoma cell line showed 8 times increase in cell number after 4 days (Wannathong et al. 2016). Clamydomonas has also been used for expression of Human immuno virus protein. This protein has paved a way for the development of microalgae based oral vaccine for AIDS (Barahimipour et al. 2016).

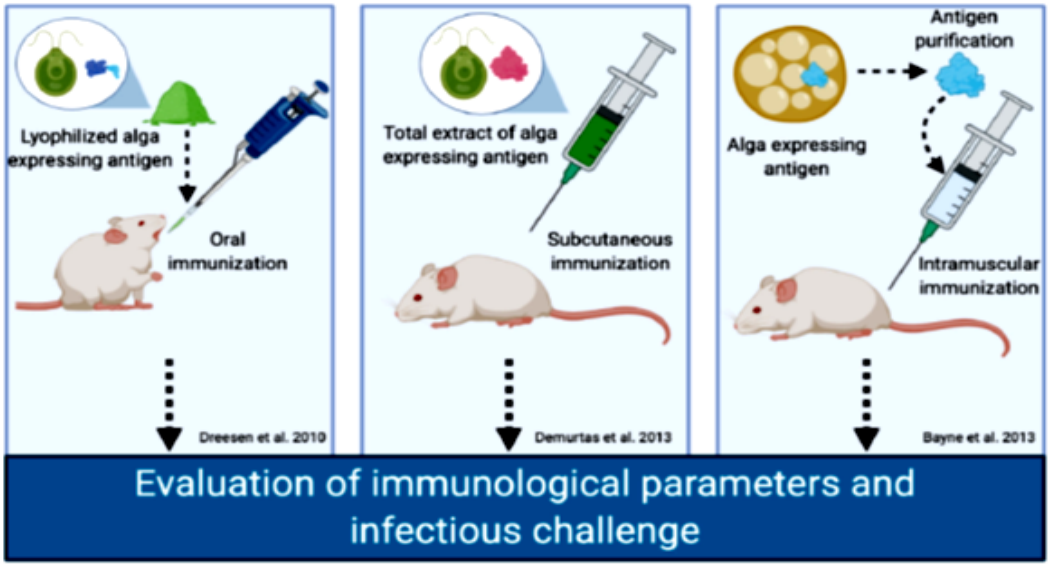

Fig. 2: Examples of evaluations of microalgae-made vaccines under different route schemes (Ramos-Vega et al. 2021).

1.15. Biodiesel

Microalgae being photosynthetic bodies are excessively rich in oil and biodiesel can be obtained by using suitable technology. Microalgae lipids possess two times more energy than other lipids. Microalgae require less space and energy in producing biomass than other crops hence are of great interest for replacing bioethanol. If oil palm can be grown 50% of transport fuel needs can be met while using only 24%of total cropland (Metzger and Largeau 2005). But oil crops cannot completely replace petroleum needs. If microalgae are for biodiesel production only 1 and 3% of cropland would be used to meet 50% transport fuel needs. Microalgae have certain advantages as well. Microalgae double their biomass within 24 hours (3.5h during exponential growth). Microalgae are also rich in oil which can exceed 80% by weight of dry biomass. Oil levels of 20–50% are quite common (Spolaore et al. 2006).

Oil production in microalgae depends on oil content of biomass and their growth rate. More oil producing algal species are the need of time. Different species produce different types of complex oil and hydrocarbons. Among all the oil production from different species of microalgae is not good fit for bio-diesel production but suitable oils are more common. Microalgae potentially a good source of natural organic carbon source such as sugar, can be used to make biodiesel (Ratledge and Wynn 2002; Guschina and Harwood 2006).

1.16. Biosafety Considerations

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In the European Union, all the genetically modified organisms and their products prior to their release in market should get an approval. There is a guidance protocol prepared by Europe Food Safety Authority (EFSA) in 2006 (Wijffels 2015). Theses containers are mostly in closed conditions in which genetically modified algae can be grown, stored, transported, destroyed, and then disposed of in the environment. The contained growth of microalgae can take place in tubular reactors, closed reactors and polyethylene. These advancements are intended to permit proficient development and the reaping of vast amounts of micro algal biomass (van der Vlugt 2020).

1.17. Risk Assessment

Risk assessment of genetically modified microalgae can be checked by testing their effect on animals, plants, humans, and environment. Risk assessments predict the toxicity and associated allergenicity and potential hazards of horizontal gene transfer (HGT) (Gressel et al. 2014). Though, to discourse this potential risk, even on the off chance that it is extremely occasional, the utilization of choice methods based on endogenous metabolic markers that supplement a missing movement in an altered beneficiary strain would be invaluable over the utilization of qualities that present resistance to herbicides or antibiotics. Evacuation of the selection markers is additionally suggested (Monier et al. 2009; Beacham et al. 2017).

1.18. Exposure Assessment

On commercial scale, the development of micro algae may cause their spillage to natural ecosystem so an exposure assessment is needed to analyze the route by which these genetically modified algae can interact with natural ecosystem. For exposure assessment digital PCR technique can be used to detect the presence of GM micro algae strains in environment (Kumar 2015).

Conclusion and Future Perspectives

Algae form the base of plant evolutionary tree. It can grow in normal as well as under harsh conditions. Algae can provide biomolecules and chemicals to biotechnology industries. They also provide food to the aquatic animals. They are the powerful source of bio medical molecules such as drugs, vaccines and antibodies (Hallegraeff 2003). Recently a complete carotenoid pathway was developed for *Xanthophyllomyces dendrorhous*, which depends on efficient expression of transgenes in microalgae (Gressel et al. 2014). Foreign gene can be expressed by integrating in chloroplast or nuclear genome. These foreign genes are loaded with desired signals that cause the synthesis of desired proteins. The expression of the genes of interest in microalgae is somehow difficult due to epigenetic mechanisms but recently these problems are being overcome. For the use of microalgae in basic and applied research, better protocols are being developed for knock-in and knock-out of gene by RNAi and CRISPR/Cas system. In conclusion, the change of frameworks for inducible expression is likewise of extraordinary significance, since it will enable us to control the transient expression of toxic particles. At long last, quick headways in genome sequencing, now regularly performed for all life forms, will demonstrate instrumental in propelling these objectives in the near future.

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REVIEW ARTICLE AGROBIOLOGICAL RECORDS
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ROLE OF ESSENTIAL OILS/VOLATILE OILS IN POULTRY PRODUCTION - A REVIEW ON PRESENT, PAST AND FUTURE CONTEMPLATIONS

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ABSTRACT

Nowadays, poultry production has high demand worldwide. For this purpose, performance parameters are maximized, for example, fast-growing of chicken with low usage of feed and with the better health status of the flock. This increasing demand has led to the use of many antibiotic-free products. There is an increased pressure to decrease the amount of antibiotics used as bacteriostatic or bactericidal agents for poultry, so there is an utmost need for unconventional resolutions to sustain the productivity and efficacy of poultry. Among the substitutions, essential oils (EOs) have a prodigious potential and are usually thought to be natural, free from hazardous deposits and chemicals, and less toxic. EOs are plant-based extracts, and there are about 3,000 known EOs, out of which 300 are identified as useful and commercially important. It is proven that EOs have abundant in vitro and in vivo research to yield special effects on numerous pathogens, including bacteria, viruses, fungi, and parasites. The current review provides information on the fundamentals of EOs, the anti-oxidation and immunomodulatory characteristics, the growth-promoting effects, and the activities of EOs against a variety of pathogens in animals/poultry.

***Keywords*:** Essential oils, Broiler/layer, Immunity, Antibiotics, Pathogens

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1. INTRODUCTION

In modern years, natural substances such as biologically active plant elements are usually considered better and used in the invention of drugs and foundations in agriculture especially in the European states like Japan and USA (Jafari et al. 2011). Some of these natural substances are known as EOs (also as volatile oils) which are aromatic in nature and are oily extractions of different parts of plants e.g., roots, flower, bud, seeds, leaves, bark and wood etc. (Nazzaro et al. 2013). There are numerous characteristics of volatile oils provided by the literature for example immunostimulatory, antiviral, digestive stimulant, antibacterial, antifungal and antioxidant (Solorzano-Santos and Miranda-Novales 2012; Bharti et al. 2012; Mahboubi et al. 2013; Bento et al. 2013; Alali et al. 2013; Krishan and Narang 2014; Fallah and Mirzaei 2016; Assiri et al. 2016). The biochemical composition of EOs talks about mode of action and the characteristics of oils. Nazzaro et al. (2013) described possible ways of action of the EOs and/or their elements and demonstrated the possible cell targets of their antimicrobial activity. However, each of these acts cannot be judged by distinct events but instead may be an outcome of the other event (Fig. 1). There are many differences among EOs which depend mainly on different kinds of variables i.e., composition of soil (physical and chemical), specie of plant used, maturity of plant, time of harvest, expertise used for drying, how the oils are stored and extraction procedure (Bakkali et al. 2008).

It has been demonstrated by Behbahani et al. (2019) that cumin EOs has very promising effect on *E. coli* (Fig. 3A) and *Listeria innocua* (4A). Briefly, in electron micrographs it is evident that the cells without treatment had the typical structure of themselves, displaying a striated wall for *E. coli* (Fig. 3A) and *L. innocua* (4A). In contrast, harmful effects on the structures/morphology of cell membranes have been displayed when cells were treated with the cumin EO at 12 and 24h at the MIC value for *E. coli* (Fig. 3B and 3C) and *L. innocua* (3B and C).

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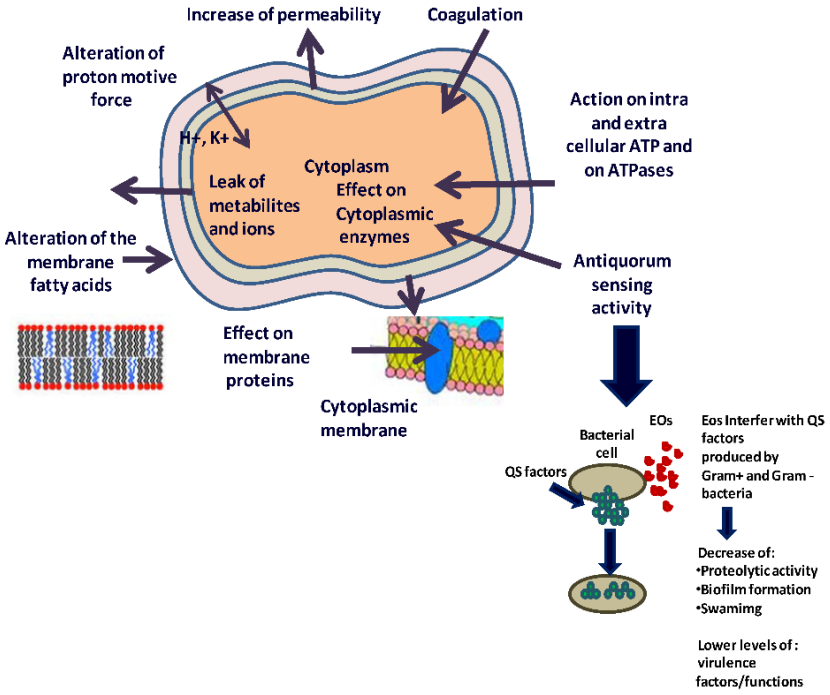

Fig. 1: Possible mode of action and target points/sites of the essential oils on a microbial cell (Nazzaro et al. 2013).

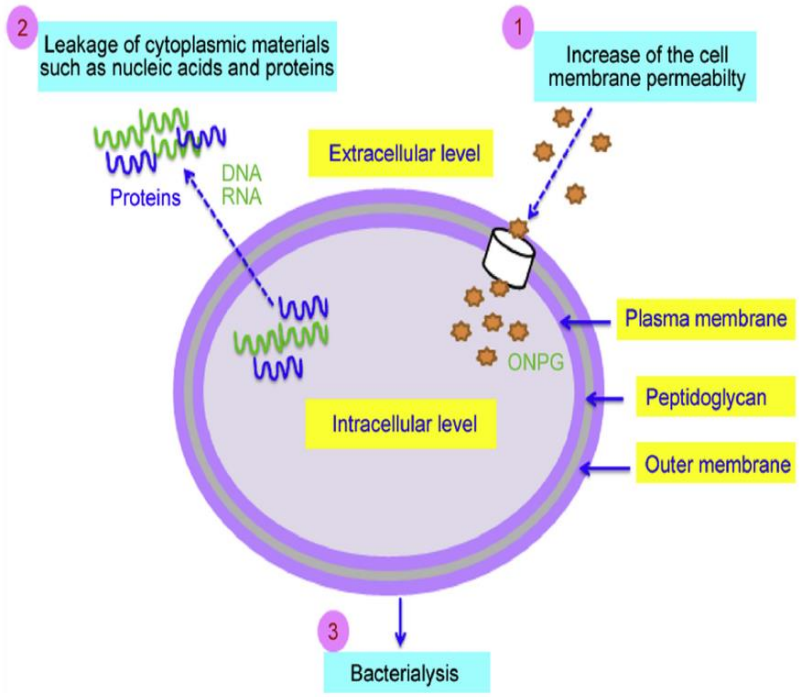

Fig. 2: Bouyahya et al. (2019) have also explained possible antibacterial actions of the EOs of *Origanum compactum* against *E. coli*. They explained that EOs increase the membrane permeability that provoke a disruption in the membrane integrity justified by the release of cytoplasm.

Microstructural level explanations that the cumin EO increases cells permeability and interrupted the cell membrane integrity (Behbahani et al. 2019). Deformed and incomplete shape was seen in treated *E. coli* escorted by the lack of cell walls. Furthermore, cumin EO altered entirely the cell membrane. Also, the shape of the cell was badly altered, and the damaged cell membrane rendered cytoplasm secretion and cell death (24 h at the MIC).

Globally, high environmental temperatures and viral infections have resulted in negative impacts on broiler production. Hence, dietary management, such as the use of natural growth promoters and plant extracts, can surge growth and immune responses though decreasing economic losses from morbidity, deaths and culling (Attia et al. 2017). The use of antibiotic growth promoters (AGP) in animal diets are banned per European Union Reg. No. 1831/2003/EC, which has increased numerous health problems and losses (Attia et al. 2016). Therefore, substitutes to AGP such as prebiotics, probiotics, EOs, and medicinal herbs have become vital (Masek et al. 2014; Hady et al. 2016).

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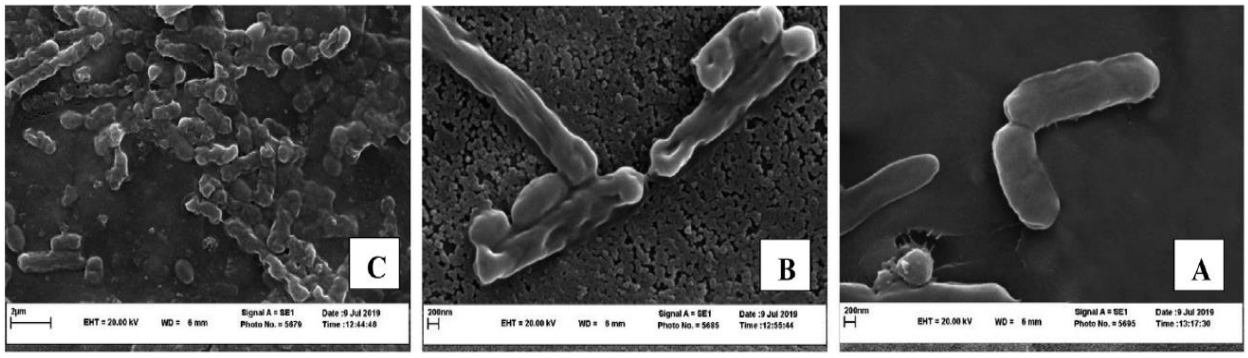

Fig. 3: Micrographs of *E. coli* cells (Scanning electron microscope: SEM): A) cells without treatment; B) *E. coli* cells treated with the cumin EO at MIC value for 12h; and C) *E. coli* cells treated with the cumin EO at MIC value for 24h (Behbahani et al. 2019).

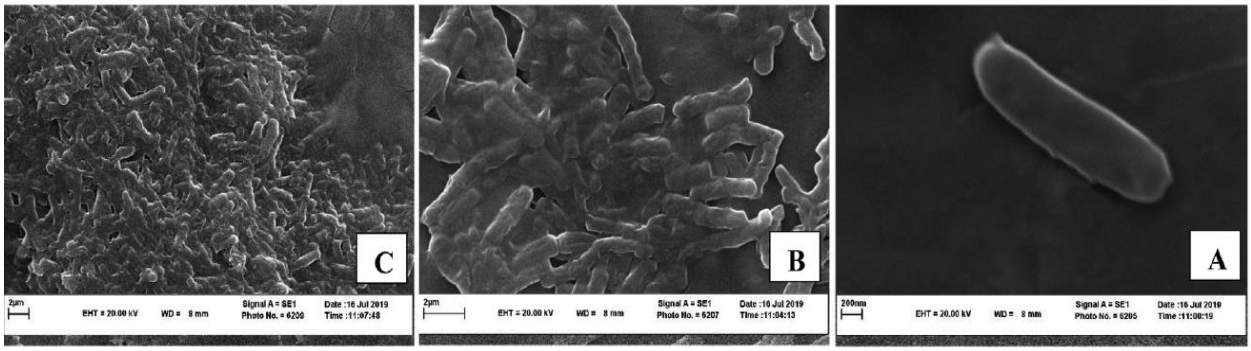

Fig. 4: SEM micrographs of *Listeria innocua* cells: A) cells without treatment; B) cells treated with the cumin EO at MIC value for 12h; and C) cells treated with the cumin EO at MIC value for 24h (Behbahani et al. 2019).

Now-a-days in poultry production, aromatic oils are in practice and used as additives in feed or drinking water due to its broad range of benefits and most of the EOs, which are being used in poultry, have been extracted from onion (*Allium cepa*), cinnamon (*Cinnamomum zeylanicum*), oregano (Origanum vulgare), ginger (*Zingiber offiinale*), eucalyptus (*Eucalyptus*), mountain savory (*S. montana*), fennel (*Foeniculum vulgare*), Australian tea tree, peppermint, lemon balm (*Melissa offiinalis*), garlic (*Allium sativum*), sage (*Salvia offiinalis*), rosemary (*Rosmarinus offiinalis*), turmeric (*Curcuma longa*), thyme (*Thymus vulgaris*), and clove (*Syzygium aromaticum*) etc. (Akbarian et al. 2014; Drăgan et al. 2014; Gopi et al. 2014; Feizi et al. 2014). Keeping in view the so much extensive research on the beneficial effects of EOs, this review has been structured to provide the information on various aspects of EOs usage in poultry production systems which are being discussed in the following sections.

The chemical composition of EOs is very diverse in nature and the main ingredient present can be part of terpenic, aliphatic or aromatic sequence. Usually, EOs comprise of ternion, seldom quaternary compounds. These volatile compounds are made up of esters, phenols, terpenes, aromatics, volatile acids, ketones, and aldehydes etc. It is not necessary that the part of plant which is subjected to hydrokinetics is always processed or treated after harvest (Zerkaoui et al. 2018). Commonly, fresh plants give better therapeutic action and more pleasant scent to solutions apart from lavender flowers, cinnamon and lime flowers which give better results when used dry. When dry plants are used, the lower volatile urine is frequently achieved because of chemical and morphologic modifications as a result of accumulation of grams, heating, air, probably by variation (Hariri et al. 2017; Bozhanska 2018).

Various essential oils used in the treatment or as growth promoters in poultry practice are of onion, garlic, thyme, oregano, aniseed, *Khuzestanica* Jamzad, myrtle, ginger, cinnamon, rosemary and turmeric. Most of these have been mentioned in Table 1.

2. Impacts of Different Essential Oils

2.1. Immunomodulatory effects

Viral infections are main risk to poultry industry and various possible hazards can enhance the threat of it. Immunosuppression is one of those hazards which can be rising from many causes such as mycotoxin, immunosuppressive infectious diseases, vaccine failure and unnecessary use of certain antibiotics and antibiotic

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growth promoters (Galal et al. 2016). Research shows that herbal EOs have positive effects on immune system of broilers as they improve lymphocytic activity, stimulate production of immunoglobulins, and boost up IFN-γ (Faramarzi et al. 2013; Krishan and Narang 2014; Gopi et al. 2014; Adaszyńska-Skwirzyńska and Szczerbińska 2017). The authors perceived that in intestines low number of intraepithelial cells in the proposes possible relaxation of strain ensuing from the GIT defensive mechanism. observed the high levels of IgA have been observed by the addition of 0.5g/kg thyme oil in feed (Placha et al. 2014). Significant increased antibody titers to infectious bursa disease are a good proof for better health due to thyme oil, moreover, higher globulin levels also indicate better resistance for the disease and immune response of bird (Bovera et al. 2015).

One of the commercial products of EO named as (AROMAX®) containing L-Menthol, Eucalyptus oil, Thyme oil and Mint oil was used against respiratory diseases and respiratory problems due to low management of broiler and the experiment showed reduced respiratory signs and improvement in humoral and local immune responses in broiler (Thajel and Ulaiwi 2017). Garlic has capability to augment the level of TNF, interleukins, interferon due to which it has immunomodulatory effect. Moreover, garlic can enhance antioxidant function, antigen presenting cells, and phagocytosis and secretory metabolism of peritoneal macrophages (Hanieh et al. 2010). One of the studies show that the addition of garlic in broiler feed regimens at 0.1% augmented hypersensitivity cutaneous basophilic response, enhanced antibodies response against ND virus and also upgrade the weight of bursa and spleen of broiler (Rahimi et al. 2011).

Another study concluded that a mixture of EOs of *Oreganum aetheroleum* is more effective than ciprofloxacin for treatment of *Escherichia coli* in broiler chickens. Results showed reduction in signs, mortalities, postmortem lesions, enhancement in cell mediated and humoral immune response and bacterial re-isolation (Abd El-Ghany and Ismail 2014).

2.2. Growth Performance, Feed Intake and Feed Utilization

Zhang et al. (2014) explained that volatile oils are considered as growth enhancers in poultry feed due to its growth promoting activity. Scientifically it is attributed as *Origanum vulgare*/*O. heracleoticum* and belongs to family Lamiaceae. Oils of this plant have high number of phenolic compounds i.e., 4.09% thymol and 69.55% of carvacol. Also, cymol, monoterpene hydrocarbons (Mathlouthi et al. 2012). Nieto et al. (2018) studied the effect of oregano EO and designate those birds which were fed Oregano EO was growing faster than that of those fed a Basal diet. Also, when oregano EO was provided from a commercial product named Synergy Essence (500g/ton); peroxidation of lipid was reduced in breast meat (minced) of chicken. Recent researchers showed that when EOs were administered in diet, they reduced the feed intake of broilers and the only possible reason is that EOs possess exasperating smell, which reduces the palatability of feed and bird may refuse to eat (Amad et al. 2011; Bozkurt et al. 2014).

Table 1: Some important essential oils, its active ingredients, and functions

Plant Essential Oil	Scientific names	Used part	Active ingredient	Functions
Aloe vera	Aloe barbadensis Miller	Leaf	Anthraquinones	Anti-inflammatory, immune-modulatory, anti-microbial anti-tumor, anti-diabetic, and antioxidant
Cinnamon	Cinnamomum Zeylanicum	Bark, leaves	Eugenol, phenolic and polyphenolic substances	Astringent, warming, stimulating, carminative, antiseptic, antifungal, antiviral, blood purifying
Garlic	Allium sativum	Bulb	γ-glutamyl-S-alk(en)yl-L-cysteines and S-alk(en)yl-L-cysteinesulfoxides, Alkin	Digestion stimulant, Antiseptic
Coriander	Coriandrum sativum	Leaves and Seed	EO comprised of limonene, carvone, borneol, elemol, geraniol, linalool, camphor, and flavonoids include rhamnetin, quercitin, epigenin and kaempferol	Appetizer, stomachic and carminative
Turmeric	Curcuma longa	Rhizome	Curcuminoids, arturmerone, zingiberene and curlone	Antioxidative, anticarcinogenic, antihepatotoxic, anti-inflammatory and hypocholesterolemic activities
Neem	Azadirachta indica	Leaves	Nimbidin, nimbinine and nimbin	Antibacterial, antifungal, antiviral, anthelmintic, stimulate fiber degrading enzymes, defaunating agent
Rosemary	Rosmarinus officinalis	Leaves	Volatile oil, tannin, bitter substances and resins	Anti-inflammatory, antioxidant
Amla	Embellica officinalis	Fruits	Gallic acid, ellegic acid, tannins, and Vitamin C	Potent antioxidant
Ginger	Zingiber officinale	Rhizome	Camphene, β-bisabolene and arcurcumene	Methane reducing capacity stimulant Gastric

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Cinnamomum zeylanicum bark and leaves are used for treating different diseased conditions. Active compound of this plant is phenolic and polyphenolic constituents and eugenol (Arain et al. 2018). A study was conducted in India by Chowdhury et al. (2018) to estimate the effect of EOs i.e., cinnamon bark oil (CNO), Ajwain seed oil and clove bud oil especially relating to their structures because structures of EO have greater effect in determining the role of EO as an alternative to AGPs for improvement of health and growth of broilers. The results of this study exposed that providing CNO (0.3g/kg) in diet was more beneficial than using antibiotic growth promoters in broiler chicks for improvement of gut health, status of antioxidant, blood cholesterol and immunity. On the other hand, Ajwain and clove oil did not show any considerable effect on immune status and gut health even at greater doses.

Barbour et al. (2011) studied the effect of Mentofin® which is a commercial preparation of eucalyptus-Peppermint EO. This commercial product was used to treat respiratory infection. This treatment was observed very useful in reduction of mortality, improvement of FCR in broiler birds and for elimination of signs and lesions of respiratory infection.

Du and his colleagues in (2016) challenged the broiler birds with *Clostridium perfringens* and supplemented the thymol and carvacol (both 25%) to investigate the effect of EO in intestinal morphology and on immune system of broiler. They concluded that EO has ability to lessen the intestinal lesions, to amend the intestinal histomorphology and to cause decrease in the inflammatory response. Noteworthy progresses were observed in feed intake of birds when 100 or 200mg of thyme and oregano (300, 500 and 700mg/kg) were included in feed (Kirkpinar et al. 2011). Gous (2010) recorded in broilers that the progresses in nutrient absorption and digestibility can cause improvement in growth.

Shomali and Mosleh (2019) reported about a local herb named as *Zataria multiflora* which is found in Pakistan, Afghanistan and Iran and it is used both as an old medicine and condiment. The medicinal herb and its different preparations have also revealed beneficial results in recent experiments of pharmacology. *Zataria multiflora* has also shown useful effects in health of broilers and performance.

Research by Goodarzi et al. (2013) exhibited that onion source can have a valuable outcome on broiler birds by improving its growth performance. When BLJ i.e., blend of encapsulated EOs and organic acids was supplemented, better growth of birds and healthier gut was observed in those broiler birds which were infected with necrotic enteritis and it occurred by establishment of the improved intestinal barrier function, positively modifying the microbiota of GIT and the intestinal immune responses were also regulated. Their results also recommended that addition of blend of encapsulated EOs and organic acids efficiently controlled necrotic enteritis disease after experimental coinfection with Eimeria and *Clostridium perfringens* (Pham et al. 2020).

Research was conducted to know about the effect of oregano EO along with multi-enzyme in protein diet (reduced) in broiler birds of day old. This oil was administered at the level of 200mg/kg in conjunction with multi-enzymes (400mg/kg) which resulted in reduction in mortality, increase in the body weight and better/improved FCR (Dafade et al. 2019).

2.3. Feed Conversion Ratio

Pimpinella anisum L. is the generic name for aniseed plant. It is aromatic and typically found in Pakistan, Iran, Turkey, India, and other countries which are appropriate for the growth of this herb. Its active component is anethole as well as other chemicals i.e., estragole, eugenol, methyl chavicol, and anisaldehyde (Ciftci et al. 2005). When anise EO was added at the rate of 400mg/kg, FCR was shown to be increased by 12% (Ciftci et al. 2005). On the other hand, thyme oil enhanced the food conversion ratio when added at level of 100 and 200mg/kg (Bölükbaşi et al. 2006).

An experiment was performed in broiler chickens for checking the effects of thyme oil. The results showed that the feed with thyme oil at the rate of hundred milligram per kilogram caused noteworthy weight gain, upgraded feed conversion ratio, livability, and turnover in broiler production system (Wade et al. 2018). Thyme belongs to genus Thyme, species vulgaris and family Lamiaceae. It is a medicinal plant and used for spices all over the world. This herb is greatly accepted in dry weathers and hot summer. EO of this vegetation are present in numerous glandular hairs in many forms and formulae and has the property to evaporate when any damage is occurred to its glandular hairs. It causes aroma which is strong in nature and this fragrance is responsible for attracting humans towards this plant which in turn extract its oils for many purposes (Stahl-Biskup and Saez 2002). Thymol and carvacol are main components of this herb and make 20 to 55% of thyme oil extract (Sengul et al. 2008).

Research was planned to show that when 200ppm of extract of oil (cinnamon and thyme) is added in the feed of broiler birds; it significantly increased the feed conversion ratio and weight (live bird) during the period of six weeks. Moreover, it also caused the reduction in ratio of high-density lipid and cholesterol level in blood. Hence it can be verified that oils of herbal plants might be useful for promoting the growth of birds in poultry production and enhance the efficacy of digestive system (Al-Kassie 2009).

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2.4. Antimicrobial and Anticoccidial Activity

Coccidia infection which is caused by protozoan parasite is a serious threat to poultry industry and a great interest is seen to control this disease by using EOs which in turn reduce the intestinal lesions in broiler chicks and a smaller number of oocysts are observed in feces (Barbour et al. 2015; Zhai et al. 2018).

Many *in vitro* studies suggested the antimicrobial activity of essential oils and among these EOs carvacol and thymol have greater antimicrobial activity for pathogenic enteric bacteria like *Salmonella typhimurium* and *E. coli* (Alagawany et al. 2015; O'Bryan et al. 2015). It is noteworthy that Gram -ve bacteria have more tolerance towards the properties of EOs as that of Gram +ve bacteria and it might be possible because of presence of hydrophilic elements in the external membrane (Brenes and Roura 2010; Giannenas et al. 2013; Seow et al. 2014).

When the immune response was amended; concentrations of pathogen was also affected (Diaz-Sanchez et al. 2015). Layer hens showed better antibody titer levels to infectious bursal disease and Newcastle disease when Heryumix was included in their feed (Özek et al. 2011). But in other study of Heryumix, the immune response (humoral) of layer was not enhanced during heat stress (Bozkurt et al. 2012).

Abbas et al. (2017) conducted a study to check the effects of EO *Camellia sinensis* on broilers for coccidiosis and the results were amazing because this oil could cause immunomodulatory property in birds against avian coccidia. The recent study inspected the *in vitro* antimicrobial action of EOs obtained from different plants and herbs like lemon, peppermint, and cinnamon etc. against subspecies of salmonella like *Salmonella enteritidis* and *S. typhimurium* which were already isolated from poultry birds. These essential oils were good for disinfection of sheds but not for treatment. Additionally, these volatile oils prevented the establishment of pathogens in intestine, when added in feed (Ebani et al. 2019).

It was concluded in a study that vital oils of *Cinnamomum zeylanicum and Eucalyptus globulus* have outstanding activity against Salmonella. It is suggested that these plant extracts may be brought into marketing as a substitute of antibiotics for the control and eradication of bacteria i.e., *Salmonellae enteritidis* and *S. gallinarum* in poultry (Yasmin et al. 2020). If we talk about the antimicrobial property of EO; it mainly depends upon many factors like lipophilic property, chemical composition, and functional group of oils. Terpenes are the most prominent feature found in these volatile oils accompanied by hydrocarbons. Cinnamon oil has linalool, eugenol etc. while Eucalyptus oil has carvone, and eucalyptol (Unlu et al. 2010; Gouveia et al. 2012).

2.5. Anti-oxidative Activity

Poultry and its products are predominantly disposed to oxidative stress and deterioration because of their high amount of polyunsaturated fatty acids. Khan et al. (2012) reported that thyme oil is used as oxidant reduction in poultry meat and eggs. This antioxidant activity might be attained by phenolic group of essential oil. The carvacol is another antioxidant nutrient which prevents the broilers from free radicals and hazardous compounds accumulation (Brenes and Roura 2010; Alagawany et al. 2015). Ginger (*Zingiber officinale*) is a rhizomic portion of this plant. It is used as a flavor and medicine too. It has *in vitro* antioxidant activity and antidiabetic (Morakinyo et al. 2011).

Myrtus communis which is from Myrtaceae family and subfamily Myrtoideae) is a medicinal plant which grows annually. Also, consumed for food and spices. Iran, Morocco, France, and some other countries are origin of this magical plant. Flavonoids and tannins are found in the leaves (Romani et al. 2004). The plant *Satureja Khuzestanica* belongs to the same family as oregano and thyme. This is mainly found in Southern Iran where it is used as ethnomedicine. It acts as an analgesic and antiseptic. It also has antioxidant property (Abdollahi et al. 2003).

Nobakht et al. (2017) did an experiment on broilers for a period of 42 days and used an extract of *Zataria multiflora* at the rate of 0.5% of feed. This resulted in reduced concentration of malondialdehyde along with increase of an enzyme in serum known as glutathione peroxidase. It was also observed that when *Zataria multiflora* was included in the diet for last 14 days of rearing, the MDA concentration gave similar results.

In alternative study, Javan et al. (2012) exposed that when broilers were fed with diets comprising 100, 200, and 400mg/kg essential oil i.e., *Zataria multiflora* for about forty-two days decreases malondialdehyde content of breast chicken meat. In a study by Kavoosi and Rabiei (2015), it was shown that thymol rich *Zataria multiflora* EOs exhibited better antioxidant activity. Onion oil was verified to have an antioxidant property with few anticancer characteristics (Ramos et al. 2006).

Another fascinating topic talks about the antioxidant traits of different EOs in chicken. It is seen that these EOs cause the reduction in peroxidation of lipids in the muscles of poultry (chicken). Thigh muscles are more prone to oxidation because of the high level of fatty acids (polyunsaturated). The oxidation of these Fatty acids yields malondialdehyde, peroxides, oxysterols, and lipids (Tongnuanchan and Benjakul 2014). Rosemary belongs to the family Labiatae which has genus *Rosmarinus* and *spp. officinalis*. This herb has maximum antioxidative action (Estevez et al. 2007). Some of the most significant biological chemicals extracted from this plant are rosmarinic acid, carnosol, antioxidants carnosic acid and camphor (Alagawany et al. 2017).

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2.6. Non-antimicrobial of EOs in Poultry

Now-a-days, EOs have been used in poultry for getting benefits for non-pathogen settings (Diaz-Sanchez et al. 2015). In one study when EOs were added to poultry feed; its digestibility was amended (Williams and Losa 2001). A commercial mixture of EOs is available known as CRINA® (Switzerland) which contain piperine, thymol and eugenol and when this blend was fed to broiler birds at level of 50mg/kg; level of trypsin, maltase and amylase was shown to be improved as compared to control (Jang et al. 2004; Jang et al. 2007).

2.7. Anti-viral Effects

Awaad et al. (2016) investigated a study on broiler chickens and concluded that the EOs mixture of peppermint and eucalyptus has controlled velogenic Newcastle disease virus vVND disease in a significant manner and it was found to enhance the immune response against this challenging virus which is causing great distressing economic effect on poultry production system.

Their efficacy in modifying the immune system of compromised birds after giving infection with Infectious Bursal Disease Virus and vaccination for Infectious Bursal Disease Virus as compared to control groups (untreated) was also obvious (Awaad et al. 2009; Awaad et al. 2010). Another study (Saderi and Abbasi 2011) reported that adenoviruses can be controlled by the used of thyme oil. Oregano oil along with its component "carvacrol" have been proved to be advantageous against Rotavirus (Pilau et al. 2011; Gilling et al. 2014) and enteroviruses (Sánchez et al. 2015). Ma and Yao (2020) have reviewed possible action of essential oils on the viral lifecycle (Fig. 5). According to them, viruses attach, penetrate, and enter the host cell, where genetic substances are replicated, followed by the creation and release of new virions (Schnitzler et al. 2010). EOs affects the virus particles at intercellular (absorption/binding) as well as intracellular (at penetration, uncoating, genome replication of viruses) stages for inhibition of the viral infectivity.

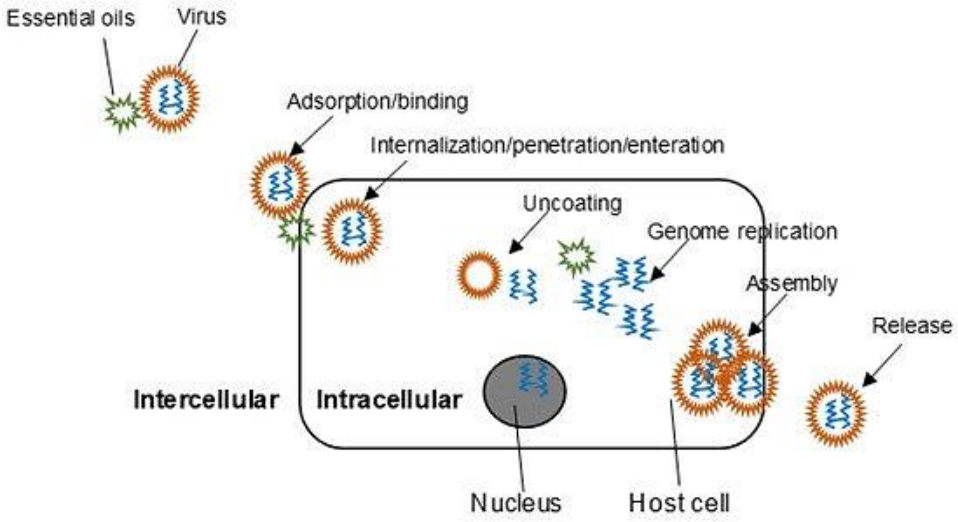

Fig. 5: Possible targeting sites of essential oils during viral lifecycle (Ma and Yao 2020).

2.8. Effects of Onion and Garlic Oils

Onion and garlic both belong to same genus Allium and many studies have improved that these both have important role in treating innumerable diseases. They are full of quercetin and compounds of sulfur. Onion belongs to Liliaceae family and has scientific name as *Allium cepa*. It is full of copper, magnesium and potassium and comprises of traces of sugar, vitamins, and fat (Gabor et al. 2012). Garlic contains high levels of potassium, phosphorus, zinc, and sulfur; medium concentration of vitamin A and C as well as selenium; and low levels of sodium, magnesium, calcium, manganese, iron, and B-complex vitamins. In addition, many compounds have been identified and isolated from garlic extracts including 33 sulfur compounds (Agarwal 1996). Garlic belongs to species *sativum* and is used as a spice in foods as well as a medicine for several years (Hanieh et al. 2010).

A data was presented about the effects of onion and garlic oils in quail and showed that onion oils act as good product for reducing the level of sugar in blood and body weights. It is also recommended that oils (onion) lower the risk of heart diseases. There are variable studies on the effects of garlic extract as a hypoglycemic role so more work and research are required to know about the facts (Abduljabbar and Abdoulrahman 2018). Garlic is beneficial treatment for different kinds of medical illnesses and is full of bio actives compound. Garlic comprises an amino acid

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having sulfur called S-allyl cysteine. It has a crucial part to act as an antioxidant (Saravanan and Ponmurugan 2010). Derivatives of garlic have proved that it is responsible for good feed conversion in broilers. Also, the height of villi (small intestine) is increased which in turn increases the absorption (Fadlalla et al. 2010).

2.9. Effects of Essential Oils on Ectoparasites

Different synthetic insecticides are in practice to control the problem of ectoparasites. However, their regular usage can cause several other problems like development of resistance, public health concerns particularly in terms of food and water residue and environment pollution. Essential oils are quite competent green alternate of insecticides (El-Seedi et al. 2017). It has been proved by the researchers and the scientists from all over the world that EOs and phytochemicals are effective against ectoparasites. Moreover, they have ovicidal, larvicidal, adulticidal and repellent effects as well (Abbas et al. 2018).

Different essential oils are found quite competent against a range of ectoparasites, particularly lice, mites and ticks. Their efficacy has been proved by different exposure routes like immersion and physical contact with EO treated surfaces, even their trace amount (vapors) was found very efficacious in spray formulation (Bakkali et al. 2008). Generally, the insecticidal activity of these oils can be attributed to their neurotoxic and suffocative effect against parasites. For example, terpinen-4-ol is a monoterpenoid found in tea tree oil at high concentration, inhibited the arthropod acetylcholinesterase, an enzyme essential for transmission of action potentials (Lopez and Pascual-Villalobos 2010). In addition to this neurotoxic effect, the hydrophobic nature of the oils may also employ the mechanical effects on the parasite which may result in disrupting the cuticular waxes and blocking their spiracles, which leads to their ultimate death (Burgess 2009).

Poultry red mite (*Dermanyssus gallinae*) it is not only the ectoparasite of laying hen but also cause restlessness, irritation, anemia and stress in broilers, wild and aviary birds (Tabari et al. 2020). EOs from different plants sources has been used against *Dermanyssus gallinae* and considered as an effective treatment against this infection. Most commonly used EOs against poultry red mites are of Coriander (*Coriandrum sativum*), clove (*Eugenia caryophyllata*) and cade (*Juniperus oxycedrus*) (Abbas et al. 2018).

Some other ectoparasites in chicken like *Menopon gallinae* (chicken lice) and *Ornithonyssus bursa* (mite) are of great economic significance in poultry. Although they do not cause death directly, but they surely are the cause of itching, weakness and lower egg production in case of layers. This parasitic burden has been controlled to a greater extent by the EOs from citronella (lemon grass) and Ginger. Chemical constituents and active ingredients in citronella (citronellal) and ginger (terpenes and oleoresin) are mainly responsible for this effect (Vigad et al. 2021).

2.10. Anti-inflammatory properties

Terpenoids and flavonoids which are components of essential oils are known to have anti-inflammatory features and these properties reduce the absorption of prostaglandins (Krishan and Narang 2014). It is observed that other compounds which are found in EOs have pain reducing, edema relieving and anti-inflammatory characteristics e.g., 1,8-cineole (found in eucalyptus oil) and linalool from lavender oil (Peana et al. 2003). Other examples of plants which possess anti-inflammatory potential are anise, chamomile, liquorice and marigold (Srinivasan 2005). Turmeric is also called as *Curcuma longa*. Rhizome is the basic part which is used. Its active ingredients are curlone, curcuminoids and zingiberene etc. Major functions of this herb are in antioxidation, as well as anticancerous, antihepatotoxic and anti-inflammatory (Vinus et al. 2018).

2.11. Anti-fungal Properties

Volatile oils mixtures and their blends also responsible for displaying antifungal activity (Owanagh et al. 2010; Hood et al. 2010). Possible mechanisms of action of antifungal bioactive compounds in Star anise essential oil (SAO) has been described by Dwivedy et al. (2018), Neto da et al. (2019) and Yu et al. (2021). According to them, SAO leads to destruction of cytoplasmic membrane, the degradation of the cell wall, or denaturing of membrane proteins those results in ions, protein, DNA and lipid damage, glucose leakage, and finally cell death (Fig. 6). There are numerous antifungal bioactive compounds (phytochemicals) in SAO such as Trans-anethole and (R)-(+)-limonene against *Aspergillus flavus* (Aggarwal et al. 2002; De et al. 2002), Limonene against *Trichophyton rubrum* (Chee et al. 2009) and α-pinene and Linalool against *Candida albicans* (Cosentino et al. 1999).

Hammer et al. (1999) described the action of monoterpenes which are very beneficial against fungi and yeast. These plant oils are also found to be advantageous for different molds relating to genus *Aspergillus* i.e., *Aspergillus fumigatus* which is responsible for causing aspergillosis in poultry birds (Esper et al. 2014). It is proposed by Esper et al. (2014) that oregano oil can be include in feed safely for reducing the effects of aflatoxin B1. In a study, effect of tea tree EO was determined for controlling the fungal diseases and it turned out to be best for eliminating the effect of fungus i.e., *penicillium* in lentils and grapes (Chidi et al. 2020).

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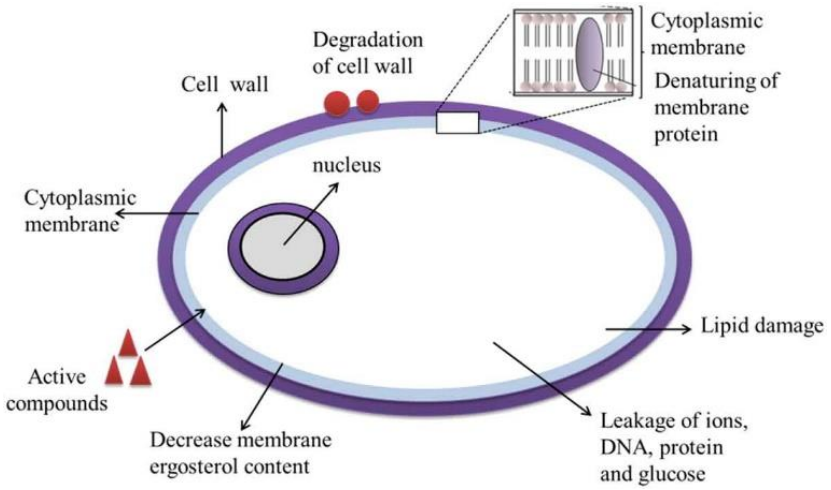

Fig. 6: Probable mechanisms of antifungal bioactive compounds in star anise essential oil (Dwivedy et al. 2018; Nato et al. 2019).

2.12. Effects on Digestive and Respiratory System

Avian gastrointestinal system is positively affected by EOs because they support the system to reestablish the balance of microorganisms and upsurge the absorption of nutrients, which may primarily be ascribed to terpenoids. These oils are very good for increasing the feed conversion as described previously (Barbour et al. 2010; Mountzouris et al. 2011).

Digestive enzymes are also produced in high quantity which result in improved absorption of nutrients and digestion. Pepsin and HCl is secreted in large amount so the better digestion of protein is accomplished (Gopi et al. 2014). Moreover, taste and smell of those substances which are present in EOs also play a vital role in the gastric juices and saliva secretion. Though, some oils cause irritation to the mucus lining of gut which then cause inflammation. It is vital, thus, to properly select and compose dose of EO.

A recent experimental study was performed to check the effects of blend of different EOs and organic acid on intestinal villi, growth of birds, blood values, and bone length of leg in broilers (Ross). A commercial feed additive was used named as Avi-protect®. In conclusion, development in intestine was seen and there was improvement in growth and metabolism of lipid in birds under study (Liu et al. 2019). Furthermore, it is suggested that thymol oil has the capability of protecting the intestinal microvilli which are accountable for the nutrient absorption, also inducing positive effect to secrete the endogenous enzymes which are consumable and prompting blood elements (Hashemipour et al. 2013).

As far as uses of EO for respiratory system is concerned, eucalyptus and peppermint oils cause the thinning of mucus so it can be easily removed from the air passages. Other oils like methanol and eucalyptol have expectorant and antispasmodic effects in host. Consequently, breathing becomes easy at the time of inflammation due to clearance of air passages (Durmic and Blache 2012).

In poultry houses, respiratory problems occur mainly during summer season because of rise in temperature and low level of humidity which in turn cause the surge in dust air. Under this type of circumstances, respiratory tract infections become more usual and severe in broiler birds due to deposition of different particles. Medicines proposed to treat the respiratory illnesses contain different oils like thyme oil and its major constitutes i.e., carvacrol and thymol. Such oils are very useful for stimulating the respiratory system and are good for smooth muscles (Edris 2007).

2.13. Other uses of Essential Oils (egg quality/biochemical/hematological)

A motivating study by Witkowska and Sowinska (2013) displayed improvement in sanitation conditions in poultry house through air sanitizers by means of thyme and peppermint oils independently as main components. An interesting study was done to explore the outcome of 7 levels (0, 25, 50, 100, 200, 400 and 600mg/kg) of plants feed additive comprising a combination of different EOs from black cumin, thyme, rosemary, fennel, and anise on growth performance of layer chicks, quality of eggshell, biomechanical characteristics and mineralization of bone in egg-laying fowls. The study was concluded by the fact that providing EOs in diet with a low or medium concentration has improved parameters involving bone, although at exceeding levels these values were badly affected in laying birds (Olgun 2016).

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The addition of EO combination @24mg/kg of feed caused significant improvement in production of eggs, efficiency of feed and it was also observed that the percentage of cracked eggs was reduced (Cabuk et al. 2006). A recent experimental study was performed to examine the positive or negative effects of housing systems and the inclusion of EOs (rosemary and cinnamon) on egg quality and performance of layer birds. Housing systems were cage and floor type while three kinds of oils i.e., zero, three hundred milligram per kilogram of rosemary, 300mg/kg cinnamon were used to check their effects on blood biochemistry, hematology, egg quality, status of immunity, oxidative level, and production of layers. The positive effects were observed on performance and quality of eggs whereas housing types did not cause any notable change in these characteristics. Parameters like level of blood cholesterol, ALT, AST, feed conversion, Ca, P, immunity, antioxidant, intake of feed, urea were found to be significantly higher and improved in treated groups (with EOs) as compared to control (Abo Ghanima et al. 2020).

A 42-days trial was conducted, and lavender oil was given in feed at the rate of 300 and 600mg/kg. Results showed that at level of 600mg/kg lavender EO, there was improvement in gut bacteria, growth rate and intestinal mucosa. Antioxidant status was better at both levels in serum and liver.

Dietary lavender EO is a better replacement for AGPs for increment of production (Barbarestani et al. 2020). A recent study proposes that essential oils which are excavated from the oranges could also be used as preserves for chicken meat. This type of method reduces the oxidation of lipids in meat (chicken) without changing the color, flavor, pH and quality (Rimini et al. 2014).

An experimental study was conducted to examine the effects of thyme and its EOs in diet. Three levels of thyme were used i.e., five, ten and fifteen milligram per kilogram and its EOs were used at different levels of 0.5, 1.0 and 1.5g/kg on antioxidant status, blood picture, growth performance and immune response in broiler chickens. This study proved to be very advantageous for different parameters of chicken (Ismail et al. 2019). The central active ingredient of thyme (aromatic plant) is thymol which act as phenolic part and is usually known to be utilized as antiseptic agent (El-Ghousein and Al-Beitawi 2009; Toghyani et al. 2010).

An experiment was conducted to assess the histopathological lesions, hematology and liver function of broilers which were given feed added with EOs e.g., *lippie rotundifolia* and lemon grass. MCV and MCH were found to be significantly lesser in negative control group (broilers). Hyperplasia of biliary duct and fibroplasia were detected in all groups with higher scores of histopathology in those broilers which were supplemented with *L. rotundifolia* oil (Santos et al. 2019). EOs of lemon grass and *Lippia rotundifolia* are also used as substitute for increasing the performance because of their antimicrobial property and are suitable for stabilizing diet (; Souza et al. 2015; Assis et al. 2017; Azevedo et al. 2017).

The current study was designed to evaluate the beneficial effects of anise, powder of curcuma seeds and fenugreek (at different levels) by feeding broiler chickens. Feed efficiency, productive performance, carcass features and few blood elements were also estimated. The results exhibited the significant effects and improvement in the FCR, live body weight and total gain, Although, the consumption of feed was not affected by the nutritional addition of these oils (Amein et al. 2019).

When the curcumin was added in feed; it regulates the secretion of bile acids and was responsible for increasing the different enzymes activity like protease, lipase, and amylase which in turn enhance the metabolism and digestion (Platel and Srinivasan 2000). Liver function also gets better by curcumin which lessens the levels of glucose in blood, triglycerides, and LDL cholesterol (Seo et al. 2008; Gandhi et al. 2011).

In a study, ginger powder was used as a natural source for improvement in growth, characteristics of carcass and the blood profile of birds (day old broilers). The dose levels were used as 0, 0.2, 0.4 and 0.6% in this experiment. The results of this study showed that there was no significant difference in weight gain of birds among different groups. However, the feed conversion ratio was better than control group. Biochemical values and hematology showed no obvious differences in dose receiving groups. So, it was determined that levels of ginger which was used in this experimental trial was not sufficient to promote the growth of chicks (Hassan et al. 2019).

Usually in broiler diets, soybean oil is used but in this experiment this oil was replaced with linseed oil (rich in omega 3) and the extract of pomegranate peel was used. Different parameters like lipid profile, fatty acids constituents, performance of birds, flavonoids and phenols were checked. Body fat was reduced due to addition of PPE and linseed oil in diet. Level of total cholesterol and triglycerides fatty acids in serum of broiler chicks was also observed at low level because of linseed oil. Providing PPE in diet enhanced the phenol and flavonoid in chicken meat (Kishawy et al. 2019).

In another study, Oregano EO was given in the feed of laying hens (commercial) to know the facts regarding this oil. The aim of this study was to check the changes in biochemical profile which were associated with liver function and metabolism of lipid and protein. Oregano EO was used @ 0, 50, 100, 150 and 200mg/kg of feed. Level of globulins and proteins was high in those groups which received dose at the rate of 150 and 200mg/kg (Migliorini et al. 2019). Also, high level of fifty-six oregano EO was responsible for increasing the cholesterol (serum levels) in broiler chickens (Basmacioglu Malayoglu et al. 2010).

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3. Essential Oils Limitations

There is no uncertainty that EOs have vast range of uses but they are also responsible for causing toxicity and unwanted effects in host (Yang et al. 2005). These volatile oils weaken the structure of cell membrane and cell wall as well as membranes of cytoplasm and cell organelles such as peroxisomes and mitochondria (Bakkali et al. 2008). EOS interrupt the mitochondrial membrane, so it causes problems in depolarization in cell via changing ion channels which in turn effect the synthesis of ATP (Vercesi et al. 1997). EOs for example thymol and carvacol have been demonstrated as toxic for the mucosa layer (intestinal cells) because of lipophilic and hydrophobic characteristic (Giannenas et al. 2003). Additionally, EOs separated from Chinese and Egyptian vegetations have been stated to produce fumigant toxicity (Fu et al. 2013). It should also consider that essential/volatile oils and their byproducts could cause hypersensitive reactions and symptoms (De Groot and Schmidt 2016).

4. Future Perspective

It is observed that environment plays an important role in poultry production. There is a correlation between environment and effects of volatile or EO and it is the need of time to thoroughly investigate it. As far as the properties of EOs are concerned, the immunomodulatory and anticoccidial activities have gained more importance and attention. The chemical structures of EOs should also be thoroughly studied to acquire the more beneficial effects from EOs.

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VERTEBRAL ANATOMIC VARIATIONS IN THOROUGHBRED RACEHORSES IN TRINIDAD

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ABSTRACT

The occurrence of lumbar vertebrae variants in horses in Trinidad is rarely reported. Parts of the skeletons of two horses of unknown age and sex that died in a horse farm in Trinidad and Tobago were brought to the Anatomy Laboratory. It was reported that specimens of fused left transverse processes of the 5th, and 6th lumbar vertebrae and a blunted left transverse process of the 6th lumbar vertebra in thoroughbred racehorses in Trinidad. It may be the first report of its kind in Trinidad and Tobago.

***Keywords*:** Anatomy, Horse, Lumbar vertebrae, Variant

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1. INTRODUCTION

The vertebral column is important for weight-bearing, locomotion, and soft tissue attachment sites. The domestic horse normally has 6 lumbar vertebrae (Stecher 1962a). The transverse processes of the lumbar vertebrae are elongated and horizontally flattened for attachment with large dorsal paraspinal muscles and ventral psoas muscle group (Haussler 1999). There are no intertransverse joints at birth, but they develop soon thereafter (Stecher 1962a). The intertransverse synovial articulations are present at the lumbosacral junction and between the last two or three lumbar vertebrae in domestic horses (Townsend and Leach 1964; Sisson et al. 1975) which help in the transfer of propulsive forces from the hind limb to the vertebral column (Haussler 1999). The importance of the thoracolumbar vertebral column in orthopedic disorders is well identified and diagnostic imaging becomes more credible in equine (Veraa et al. 2016; Spoormakers et al. 2021).

2. Case Description

Part of the skeletons of two horses of unknown age and sex that died in a horse farm in Trinidad and Tobago were brought to the Anatomy Laboratory, School of Veterinary Medicine, Faculty of Medical Sciences, University of the West Indies, Trinidad and Tobago for demonstration of the axial skeleton to year I veterinary students. The skeletons were dissected, disarticulated, boiled, bleached in 3% hydrogen peroxide, and then air-dried. Photographs were taken for documentation using a Kodak digital camera. The transverse processes of the normal lumbar vertebrae were elongated, flattened, and horizontal and gave the attachment site for the epaxial and hypaxial muscles. Normally there is a pair of intertransverse synovial articulations between the 5th and 6th lumbar vertebrae; however, there were specimens of anatomical variants in which there was a fusion of the left transverse processes of the 5th and 6th lumbar vertebrae in two specimens (Fig. 1A and 1B). The left transverse process of the 6th lumbar vertebrae was peripherally blunted in one specimen (Fig. 1B).

3. DISCUSSION

Most of the intertransverse synovial articulations of the lumbar vertebrae in the horse typically occur in pairs between the left and right transverse processes of the 5^{th} and 6^{th} lumbar vertebrae; a similar finding was in the horse (Stecher 1962b; Townsend and Leach 1964; Haussler et al. 1997; Haussler 2018).

The stabilizing function of the intertransverse joints occurs via giving resistance against the lateral bending and axial rotation of the spine, limiting the range of the lumbar motion and helping in the transfer of propulsive forces from the pelvic limbs to the vertebral column (Stecher and Goss 1961; Townsend and Leach 1964; Haussler 2018).

The current study reported that the right intertransverse synovial articulation was present in two specimens, while there was a fusion between the left transverse processes of the 5^{th} and 6^{th} lumbar vertebrae (ankylosis); similar findings were reported in the horse (Stecher 1962b; Haussler et al. 1997; Collar et al. 2015; Spoormakers et al. 2021).

There is a possible relationship between the asymmetry in the intertransverse joints and asymmetry in back motion (Faber et al. 2000). The fusion of the intertransverse joints limits their movements (Townsend and Leach

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1964). Intertransverse joint ankylosis is common either unilaterally or bilaterally and is probably not a significant cause of back pain but maybe for further adaptations to strain in horses (Townsend and Leach 1964; Haussler et al. 1997). The current case report showed that the left transverse process of the 6th lumbar vertebrae was peripherally blunted in one case which is similar to the finding in the horse (Collar et al. 2015).

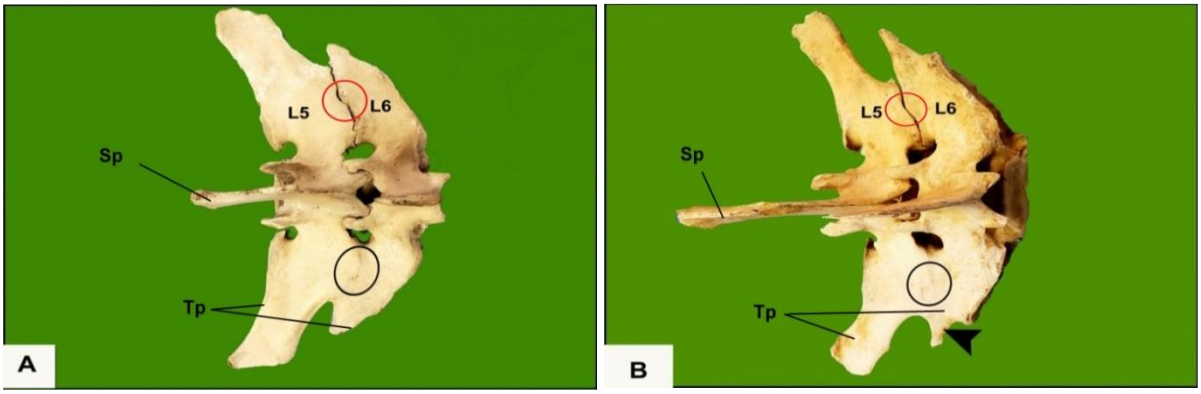

Fig. 1: A photograph showing fused left transverse processes of the L5-L6 (black oval; A and B) and a left blunted transverse process of L6 (black arrowhead). L5-5th lumbar vertebra; L6-6th lumbar vertebra; Sp-Spinous process; Tp-Transverse process; Red oval-Intertransverse joint (ITJ).

Conclusion: Knowledge of normal spinal morphology and vertebral variation is of extreme clinical importance for surgeons and radiologists for the distinction of pathologic change from normal anatomic variations.

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